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PRINCIPAL INVESTIGATOR: Kun-Liang Guan

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, CA 92093-0621

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Kun-Liang Guan

E-Mail: kuguan@ucsd.edu

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14. ABSTRACT
Mutations in TSC1 and TSC2 genes are responsible for the majority of tuberous sclerosis complex (TSC). The major function of TSC1/2 is to inhibit mTORC1. Therefore, uncontrolled mTORC1 activation is a key molecular basis for TSC and TORC1 inhibitors is being used for TSC related complication. We discovered that mTORC1 can be inhibited by intracellular cAMP. We further found that the protein kinase A (PKA) mediates the effect of cAMP to inhibit mTORC1. Overexpression of the catalytic subunit of PKA leads to reduced mTORC1 activity while inhibition of PKA by pharmacological inhibitors blocks the inhibitory effect of cAMP on mTORC1. Furthermore, we found that cAMP and PKA function to prevent mTOR lysosomal localization, which is a process critical for mTORC1 activation. Our studies reveal a potential mechanism and future research direction how PKA inhibits mTORC1. This project also connects the two major intracellular signaling pathways, cAMP and mTOR, in the regulation of cell growth.

15. SUBJECT TERMS
TSC, mTOR, cAMP, PKA.

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder characterized by the development of benign hamartomas in many organs, such as the brain, kidney, heart, skin, and eyes[1, 2]. TSC results from mutations in the tumor suppressor genes TSC1 and TSC2, which lead to an increase in cell growth and tumor formation[3]. The proteins encoded by TSC1 and TSC2, hamartin and tuberin, respectively [4, 5], form a physical and functional complex upstream of the mammalian target of rapamycin complex 1 (mTORC1). TSC1 stabilizes TSC2 [6, 7], and TSC2 acts as a GTPase-activating protein (GAP) to promote GTP hydrolysis activity of the small GTPase Rheb [8-13]. GTP-bound Rheb activates the mammalian target of rapamycin complex 1 (mTORC1) [14, 15] which in turn accelerates cell growth, metabolism and tumor development. Mutations in either TSC1 or TSC2 induce benign tumor growth mainly due to constitutive mTORC1 activation by Rheb [1]. The uncontrolled mTORC1 activity contributes significantly to the pathogenesis of TSC. mTORC1 is regulated by a wide range of signals, including growth factors, amino acids, cellular energy status, and stress[16]. Among all the stimuli that activate mTORC1, amino acids serve as the key signal for mTORC1 activation[17-19]. The major goal of this proposal is to determine the mechanism(s) of cAMP in regulation of the mTORC1, with particular focus on the signaling cross talk between cAMP and amino acids. Moreover, we aim to determine the function of GPCR signaling and cAMP in mTORC1 regulation and the functional significance of this crosstalk in TSC pathogenesis.

Keywords

Tuberous sclerosis complex, TSC, TSC1, TSC2, mTOR, mTORC1, amino acid, cAMP, GPCR, Tumor, phosphorylation, kinase, PKA

Overall Project Summary

Specific Aim 1: To elucidate the molecular mechanism of cAMP and PKA in mTORC1 regulation. We hypothesize that PKA specifically blocks amino acid-induced mTORC1 activation, possibly by phosphorylating key components in the amino acid signaling cascade. To test this hypothesis we plan to test the following specific subaims: 1.1) To determine whether cAMP inhibits amino acid transport; 1.2) To test whether cAMP inhibits mTORC1 lysosomal localization; 1.3) To investigate the effect of cAMP on Rag activation by amino acids; 1.4) To elucidate the effect of cAMP on Rag localization or Rag-Raptor interaction; 1.5) To determine the effect of cAMP on mTORC1 activity; and 1.6) To investigate the biochemical mechanisms of PKA in Forskolin inhibition.

1.1) To determine whether cAMP inhibits amino acid transport

To test whether PKA inhibited the transport of amino acids into the cell, we measured leucine transport in the presence or absence of Forskolin. Cells were starved of amino acids for 50 minutes and then re-stimulated with medium containing amino acids and ^{3}H-labeled leucine. After incubation (between 0-60 minutes) of ^{3}H-labeled leucine, cells were washed and amino acid uptake by the cells was quantified via scintillation counting. We did not observe any significant change in leucine uptake in the absence or presence of Forskolin (data not shown). Other amino acids besides leucine activate mTORC1, such as glutamine and arginine[20, 21]. We are currently testing the effect PKA has on glutamine and arginine uptake in multiple cell lines. Furthermore, we have screened and determined which amino acids specifically modulate mTORC1 activity. We are now specifically looking at which amino acid(s) PKA can inhibit in signaling to mTORC1.

1.2) To test whether cAMP inhibits mTORC1 lysosomal localization

Preliminary data in HeLa cells demonstrates that Forskolin-induced activation of PKA may block mTORC1 lysosomal localization and activation (Figure 1). Consistent with previous studies we observe a high degree of mTOR localization at the lysosome in the presence of amino acids (3rd column)[22]. mTOR staining is illustrated in green and LAMP2 (lysosome marker) is shown in red. In contrast, under amino acid starvation conditions mTOR is dispersed throughout the cell and not at the lysosome (4th column). In cells treated with Forskolin 1 hour prior to amino acid stimulation, mTOR is not localized with LAMP2. Taken together, these preliminary results suggest that PKA is somehow blocking mTORC1 lysosomal localization and subsequent activation. To confirm these preliminary findings we are currently performing a stimulation time course of amino acids in both HeLa and MEF cells, and in the presence and absence of Forskolin. To ensure that the localization is indeed mTORC1 and not mTORC2 (mTOR is present in both complexes), we are currently performing similar immunoflourescence experiments with antibodies specifically recognizing raptor, which is an mTORC1 specific subunit.

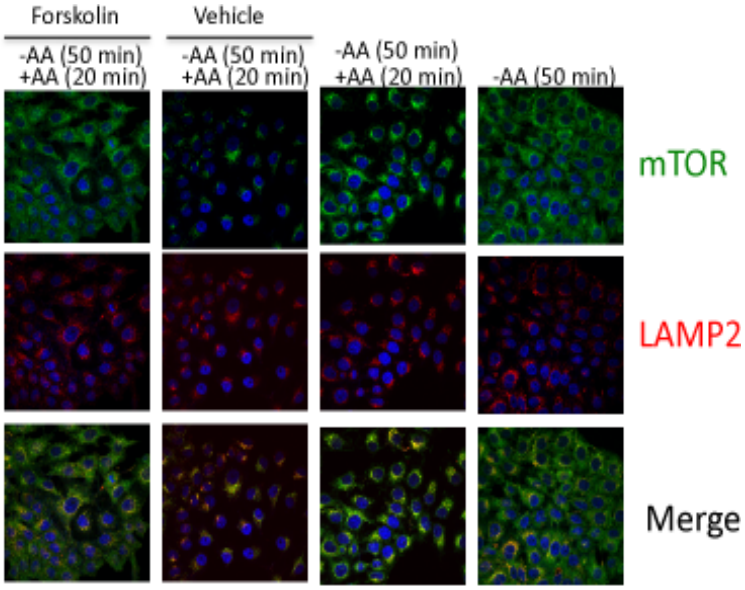

Figure 1: Localization of mTOR in cells treated with Forskolin.

The PKA inhibitors H89 and mutant R-subunit are now being tested to see if we can reverse the inhibition Forskolin has on mTOR lysosomal (LAMP2) localization. We anticipate that if we inhibit PKA prior to Forskolin treatment mTOR will still translocate to the lysosome in response to amino acids. In addition, we are testing the effect of physiological cAMP-PKA modulation on mTORC1. We are treating cells with epinephrine and subsequently analyzing mTORC1 lysosome localization and activation.

1.3) To investigate the effect of cAMP on Rag activation by amino acids

The role of cAMP through PKA on Rag GTPase activation by amino acids has yet to be determined. We are now in the process of looking at the guanine nucleotide loading of the Rag GTPases in the presence and absence of Forskolin. Although, we hypothesis that mTORC1 inhibition by PKA may not require the Rags. Our laboratory recently generated RagA/B knockout (KO) mouse embryonic fibroblast (MEFs). By utilizing these MEFs, we identified another amino acid signaling pathway to mTORC1 in the absence of the Rags (*manuscript in revision*). Interestingly, Forskolin inhibited mTORC1 in cells deleted of the Rag GTPases similar to that of control cells. The novel amino acid signaling pathway that we identified doesn't require the Rags or the Ragulator, but it does require the v-ATPase and lysosome.

We have preformed some studies looking at the effect PKA has on the v-ATPase. Recently, the v-ATPase has been shown to be an important player in amino acid-induced mTORC1

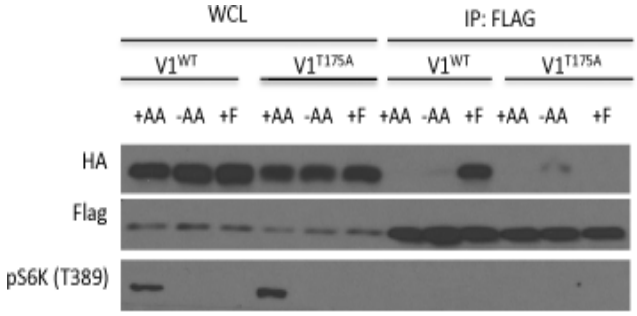

Figure 2: PKA interacts with the wild-type V1A subunit under Forskolin treatment.

activation, playing a role between amino acids and the Rags[23]. We also see that the v-ATPase is important in a Rag independent amino acid signaling pathway (*manuscript in revision*).

Interestingly, it was recently reported that PKA can phosphorylate and regulate the localization of V1 subunit of the v-ATPase[24]. The phosphorylation site by PKA was mapped to be threonine 175. Co-immunoprecipitation studies revealed that the catalytic subunit of PKA (HA PKA-Cat) can directly bind to the V1A subunit (Flag V1A; Figure 2). Interestingly, the binding between PKA and the v-ATPase V1 subunit appears to be dependent of Forskolin treatment and the reported PKA phosphorylation site, threonine 175. We are further exploring the v-ATPase, specifically the V1 subunit, as being a potential PKA substrate. We are also interested in the impact this PKA site has on mTORC1 activation and cell growth.

1.4) To elucidate the effect of cAMP on Rag localization or Rag-Raptor interaction

The role of PKA in the interaction between the rags and ragulator has not yet been determined. We are currently investigating this along with whether or not Rag lysosomal localization is altered with the addition of Forskolin treatment. In addition, we are currently testing if the binding between mTORC1 (raptor subunit) and the Rag GTPase is altered in Forskolin treated cells. Because of the preliminary confocal localization data in 1.2) we anticipate less Raptor-Rag interaction in Forskolin treated cells.

1.5) To determine the effect of cAMP on mTORC1 activity

We are currently investigating this subaim. Specifically, to role out Forskolin is controlling a phosphatase instead of mTORC1 kinase activity, we are observing other mTORC1 substrates via Western blot analysis using a specific phospho-antibody (Ulk1 S757) [25]. Furthermore, we are performing mTORC1 *in vitro* kinase assays in the absence or presence of Forskolin as previously described, to directly monitor mTORC1 activity[26, 27].

1.6) To investigate the biochemical mechanisms of PKA in Forskolin inhibition

We have ruled out the Rag GTPases as direct PKA substrates via *in vitro* kinase assays (data not shown). We are currently looking at mTORC1 and the Ragulator as possible PKA substrates. Currently, our main focus is the V1 subunit of the v-ATPase as a potential PKA substrate, refer to 1.3). To better understand the functional significance of the phosphorylation of T175 on the v-ATPase subunit V1 in respect mTORC1 activation, we plan to use CRISPR based genome editing to endogenously tag the V1 subunit and introduce point mutations (T175A; phospho-defective and T175D; phospho-mimetic). By endogenously tagging the v-ATPase, we can monitor its lysosomal localization in response to cAMP levels. The endogenous knock-in point mutations will demonstrate the significance of the PKA phosphorylation site on amino acid signaling to mTORC1. Taken together, our results may uncover cross talk between mTORC1 and PKA at the lysosome, specifically involving the v-

ATPase.

Specific Aim 2: To study the biological functions of mTORC1 regulation by cAMP-PKA. mTORC1 promotes translation and inhibit autophagy, leading to cell growth and proliferation. This specific aim is designed to test the functional significance of mTORC1 inhibition by cAMP-PKA in the regulation of translation, autophagy, and cell growth. By increasing cAMP by clinical drugs, we will test the role of growth inhibition in TSC mutant cells. To test this hypothesis we plan to test the following specific subaims: 2.1) Regulation of mTORC1 dependent protein translation by cAMP; 2.2) To determine the function of cAMP on autophagy induction; 2.3) To investigate the function of cAMP in cell growth; and 2.4) To test the effect of cAMP elevating drugs on the growth of TSC mutant cells.

We are preparing reagents and cell lines for experiments in aim 2.

Key Research Accomplishments

Nothing to report

Conclusion

This proposal is currently in progress to understand the molecular mechanisms of the crosstalk between to major signaling pathways, mTORC1 and cAMP-PKA. The cAMP-PKA pathway is modulated by a number of physiological hormones via G-protein coupled receptors (GPCRs). GPCRs are the target of approximately 40% of medicinal drugs. In addition, the cAMP-PKA pathway was been implicated in inhibiting growth. mTORC1 integrates numerous stimuli to promote cell growth. Both pathways have found a way to coordinate with one another in order to achieve the appropriate size of a cell. At a molecular level it is important to delineate the mechanisms involved in the crosstalk of these pathways, in order to promote the testing of already approved drugs (target of GPCRs etc.) for TSC patients. This proposal may also result in the discovery of a new drug target for the treatment of TSC. There are currently many drugs in clinical trials modulating the cAMP-PKA pathway that may also benefit TSC patients.

We have shown that cAMP inhibits mTORC1 in a mechanism likely to be independent of amino acid transporter, at least for leucine. Furthermore, we demonstrated that PKA is mediating the effect of cAMP on mTORC1 inhibition. Our mTOR localization studies show that cAMP inhibits the lysosomal translocation of mTOR in response to amino acid stimulation. These data provide important new insights into the mechanism of mTORC1 inhibition by PKA. Our future efforts will focus on how PKA blocks the mTOR translocation. Another interesting finding in the past year is that PKA physically interacts with the lysosomal ATPase V1 subunit. Importantly, the interaction between PKA and ATPase V1 is stimulated by forskolin, suggesting that this interaction is regulated by cAMP. Together with the finding that the Rag GTPases are not direct targets of PKA, our results strongly indicate that PKA may inhibit mTORC1 by directly interacting with and phosphorylating the V1 subunit of ATPase.

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1. Jewell, J.L. and Guan, K-L. (2013) Nutrient signaling to mTOR and cell growth. TIBS. 38, 233-242.
2. Jewell, J.L. Russell, R.C., and Guan, K-L. (2013) Amino acid signaling upstream of mTOR. Nature Rev. Mol. Cell. Biol. 14, 133-139.

Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

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Appendices

Two publications.

Review

Nutrient signaling to mTOR and cell growth

Jenna L. Jewell and Kun-Liang Guan

Department of Pharmacology and Moores Cancer Center, University of California at San Diego, La Jolla, CA 92093, USA

The mammalian target of rapamycin (mTOR) is a conserved protein kinase involved in a multitude of cellular processes including cell growth. Increased mTOR activation is observed in multiple human cancers and inhibition of mTOR has proven efficacious in numerous clinical trials. mTOR comprises two complexes, termed mTORC1 and mTORC2. Both complexes respond to growth factors, whereas only mTORC1 is controlled by nutrients, such as glucose and amino acids. Since the discovery of mTOR, extensive studies have intricately detailed the molecular mechanisms by which mTORC1 is regulated. Somewhat paradoxically, amino acid (AA)-induced mTORC1 activation –arguably the most essential stimulus leading to mTORC1 activation – is the least understood. Here we review the current knowledge of nutrient-dependent regulation of mTORC1.

mTOR signaling pathway

TOR (mTOR in mammals, or also referred to as mechanistic TOR) is a conserved atypical serine/threonine protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family; however, it is a protein kinase. As its name implies, it is the target of rapamycin; a natural compound first isolated thirty-seven years ago from the bacterium *Streptomyces hygroscopicus* and soon afterwards discovered to have antiproliferative properties. Sixteen years later, genetic screening in *Saccharomyces cerevisiae* identified that mutated TOR1 and TOR2 genes conferred rapamycin resistance [1,2]. Subsequent studies in mammals uncovered mTOR as the target of rapamycin [3–5]. Analogs of rapamycin are currently in clinical trials for the treatment of various cancers. Everolimus and temsirolimus have recently been approved for late stage renal cancer [6].

Early studies in yeast have revealed TOR as a multifunctional protein kinase, and not all functions of TOR are sensitive to inhibition by rapamycin. This has led to the discovery of two distinct TOR complexes, TORC1 and TORC2 [7]. The two TOR complexes are conserved in mammals, referred to as mTORC1 and mTORC2; the former of which is potently inhibited by rapamycin [8,9]. Later studies have demonstrated that prolonged rapamycin treatment also inhibits mTORC2 assembly by sequestering mTOR in some cell types [10,11]. Growth factors control both mTOR complexes and mTORC1 is also regulated by stress and nutrients, such as AAs and glucose (Figure 1) (reviewed in [12]). This review focuses on nutrient regulation of mTORC1; additional information on mTORC2 is reviewed elsewhere [13]. mTOR is the catalytic subunit of mTORC1. Other components of mTORC1 include: regulatory-associated protein of mTOR (Raptor), which is involved in substrate recognition; mTORC1 inhibitory modulators proline-rich AKT/PKB substrate 40 kDa (PRAS40) and Dep-domain mTOR interacting protein (Deptor); and the positive mTORC1 regulator mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL) [14]. Three independent groups have shown that mTOR controls its own activation by degrading Deptor through SCF(βTrCP) E3 ligase, although the mechanistic details of these studies differ significantly [15–17].

Ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIFAE) binding protein (4EBP, also known as PHAS-1) are the two best-characterized substrates of mTORC1, which promote protein synthesis (reviewed in [18]). Although mTORC1 has been shown to regulate translation in numerous studies, the overall translational program controlled by mTORC1 has been uncertain until recently. Two global profiling studies have identified specific mRNAs whose translation is strongly stimulated by mTORC1; they encode proteins involved in translation, cell proliferation, invasion, and metabolism [19,20]. In addition to controlling protein synthesis, mTORC1 has been shown to target and control components involved in autophagy, lipid synthesis, insulin action, and ribosome biosynthesis (reviewed in [14]). High mTORC1 activation suppresses autophagy under nutrient sufficiency. Recent studies have demonstrated that phosphorylation of unc-51-like kinase 1 (ULK1) and autophagy-related protein 13 (ATG13), both subunits of the autophagy initiating kinase ULK1 complex, may represent an underlying mechanism of autophagy inhibition by mTORC1 [21–24].

Multiple upstream signals including growth factors, stress, and nutrients control mTORC1 (Figure 1) [12]. Growth factors regulate the tuberous sclerosis complex (TSC). TSC1 and TSC2 form a physical and functional TSC complex. TSC1 stabilizes TSC2 [25,26], and TSC2 acts as a GTPase-activating protein (GAP) to promote the inherent GTP hydrolysis activity of the small GTPase Rheb [27–32]. A third component of TSC, Tre2-Bub2-cdc16 (TBC)1 domain family, member 7 (TBC1D7), has just been identified; it is thought to promote TSC1–TSC2 interaction and TSC2 GAP activity towards Rheb [33]. Activated GTP-bound Rheb, under TSC inhibition, binds to and

Corresponding author: Guan, K.-L. (kuguan@ucsd.edu)

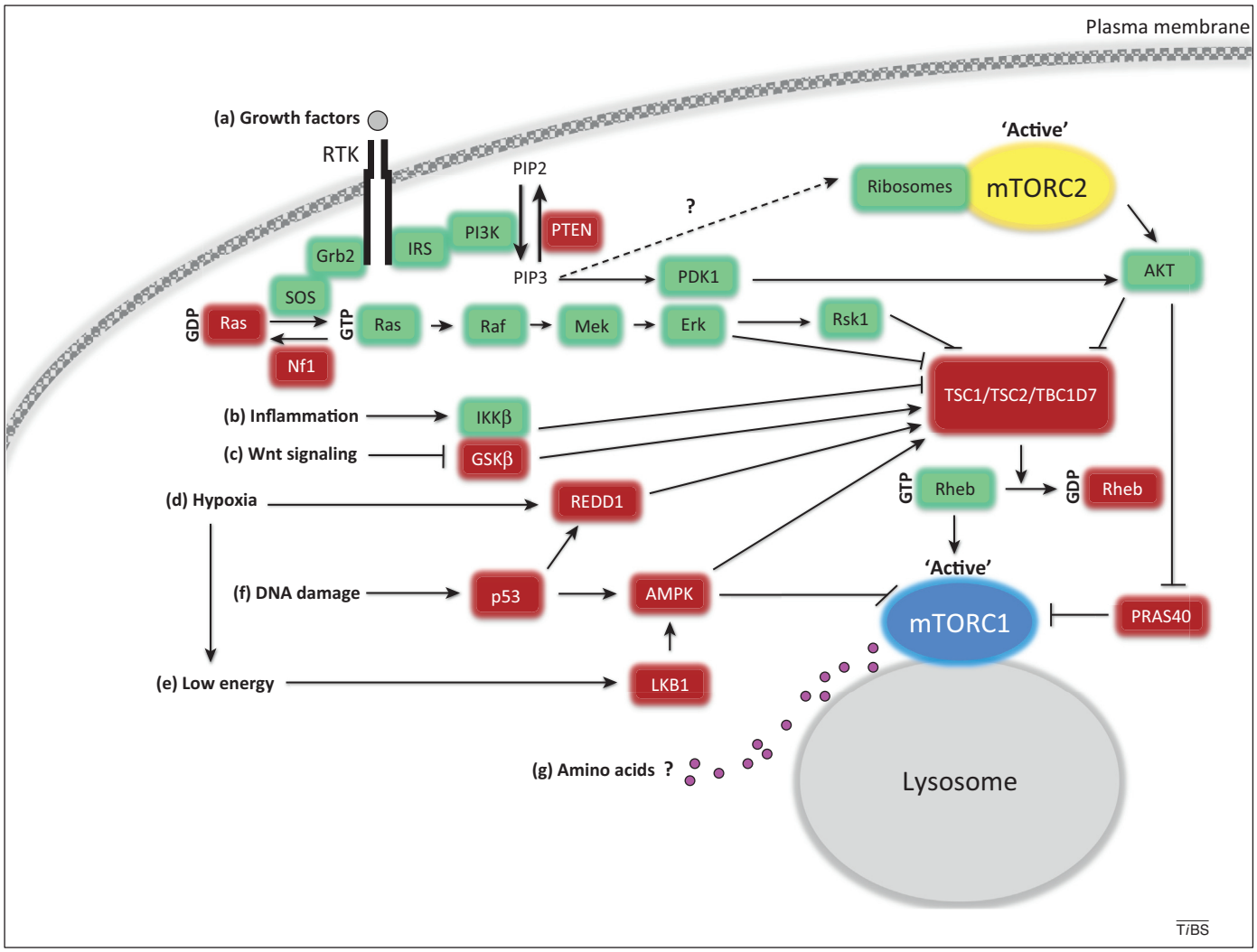


Figure 1. The mammalian target of rapamycin (mTOR) signaling cascade. mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) are both controlled by growth factors, whereas mTORC1 is also regulated by cellular energy status, oxygen, stress, and amino acids (AAs). **(a)** mTOR is controlled by growth factors through the classical phosphatidylinositide 3-kinase (PI3K)–protein kinase B (PKB; also known as AKT)-tuberous sclerosis complex (TSC) pathway and through the Ras signaling cascade. PI3K is activated and recruited to the membrane by insulin receptor substrate (IRS)-1, where it catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). AKT is recruited via its pleckstrin homology (PH) domain binding to PIP3, and is phosphorylated and activated by the phosphoinositide-dependent kinase (PDK)1. mTORC2 is thought to be activated downstream of PI3K, possibly by ribosome binding, although many unanswered questions remain. Broken arrow represents missing components possibly involved that have not been identified. Full activation of AKT requires the phosphorylation of T308 (by PDK1) and S473 (by mTORC2). The requirement for AKT phosphorylation at T308 or S473 depends on its substrate. AKT kinase activation can promote mTORC1 signaling in two ways: by phosphorylating and inhibiting TSC2 GTPase activating protein (GAP) activity, thus activating Rheb, and by phosphorylating proline-rich AKT/PKB substrate 40 kDa (PRAS40), which increases PRAS40-14-3-3 binding and relieves PRAS40 inhibition on mTORC1. The Ras pathway regulates mTORC1 through extracellular signal-regulated kinase (ERK) and ribosomal S6 kinase 1 (RSK1), which phosphorylate and inhibit TSC1/2. Tre2-Bub2-cdc16 (TBC)1 domain family, member 7 (TBC1D7), a third subunit of TSC, promotes TSC1–TSC2 interaction and the GAP activity of TSC2 towards Rheb. **(b)** IkB kinase β (IKKβ) can also inhibit TSC1/TSC2 in response to inflammation. **(c)** Glycogen synthase kinase (GSK)3-β, which is regulated by Wnt signaling, phosphorylates TSC2 and increases its GAP activity in an AMP-activated dependent kinase (AMPK)-priming phosphorylating manner. **(d)** Hypoxia promotes expression of regulated in development and DNA damage responses 1 (REDD1), which activates TSC1/TSC2, and hypoxia activates AMPK due to the failure to generate sufficient ATP. **(e)** When cellular energy is low AMPK is activated and regulates mTORC1 by phosphorylating and activating TSC2. Also, AMPK can phosphorylate regulatory-associated protein of mTOR (Raptor) and inhibit mTORC1 function. **(f)** DNA damage results in the inhibition of mTORC1 activity through the p53-dependent upregulation of REDD1 and AMPK. **(g)** AA signaling activates mTORC1 (see Figure 3). Pink circles represent AAs. Abbreviation: RTK, receptor tyrosine kinase.

potently activates mTORC1 through an unknown mode of action. In addition to growth factors, additional stimuli such as inflammation, Wnt signaling, hypoxia, low energy status, and DNA damage unite at the TSC complex in order to control mTORC1 (reviewed in [34]). Therefore, the TSC complex represents a crucial upstream regulator of mTORC1 (Figure 1).

Nutrient availability is fundamental for cell growth and the survival of all organisms. Cells respond to the amount of nutrients by triggering either energy-consuming anabolic pathways under nutrient sufficiency, or energy-producing catabolic pathways under stress and starvation conditions. mTOR orchestrates these processes; it is activated under nutrient-rich conditions and its function is blocked under nutrient-limiting conditions. mTORC1 carefully integrates these signals to control many fundamental processes involved in cellular metabolism and growth. Glucose availability and the fluctuation of energy is translated to mTOR by AMP-activated protein kinase (AMPK), which directly senses energy fluctuation. By contrast, the identification of the AA sensor or sensors and their location – extracellular, intracellular, or within the lysosome – remain unclear. Many intermediates in controlling AA mTORC1 activation have been identified including the

Table 1. Components identified to be involved in the amino acid (AA) signaling cascade to mTORC1

Component	Function	mTORC1 Activation	References
Arf	Small GTPase, vesicle transport mediator	↓	[70]
C7orf59	Component of the Ragulator complex, Ragulator is GEF for Rag A/B	↑	[55]
GCN2	Kinase, binds uncharged tRNA	↓	[79,80]
HBXIP	Component of the Ragulator complex, Ragulator is GEF for Rag A/B	↑	[55]
IPMK	Inositol and lipid kinase, binds to mTOR and Raptor	↑	[75]
LeuRS	Enzyme, charges leucine to cognate tRNA, binds to Rags	↑	[71,72]
MAP4K3	Kinase, thought to be activated downstream AA but upstream of mTORC1	↑	[88,89]
mLST8 (GβL)	Binds both mTOR complexes, necessary for mTOR-Raptor AA sensitive interaction	↑	[73,74]
MP1	Component of Ragulator complex, Ragulator is GEF for RagA/B	↑	[51,55]
mTOR	Kinase, catalytic subunit of mTORC1 and mTORC2	↑	[90]
p14	Component of Ragulator complex, Ragulator is GEF for Rag A/B	↑	[51,55]
p18	Component of Ragulator complex, anchors complex to lysosome, Ragulator is GEF for Rag A/B	↑	[51,55]
p62	Targets cargo for autophagy, binds to Rag Complex	↑	[91]
PAT1	Transporter at the lysosome involved in AA mTORC1 activation	↓	[62,65–67]
PLD	Enzyme, catalyzes the hydrolysis of PC to form PA, binds and activates Rheb	↑	[81,82]
Rab5	Small GTPase, vesicle transport mediator, inhibition of mTORC1 dependent on Rags	↓	[70]
RalA	Small GTPase, partially rescues mTORC1 when Rheb is absent	↑	[69]
RagA/B	Small GTPase, heterodimerizes with RagC/D, binds to Raptor, GTP loaded to be active	↑	[41,48]
RagC/D	Small GTPase, heterodimerizes with RagA/B, binds to Raptor, GDP loaded to be active	↑	[41,48]
Raptor	Regulatory component of mTORC1, involved in substrate recognition	↑	[73,74]
Rheb	Small GTPase, $Rheb^{GTP}$ activates mTORC1 via binding	↑	[52]
SH3BP4	Binds to the inactive Rag complex	↓	[83]
VPS34	Lipid kinase, reported to promote mTORC1 activation	↑	[82,92]
VPS39	*Saccharomyces cerevisiae* homolog Vam6 reported as Gtr1 GEF, VPS39 does not appear to be RagA/B GEF in mammals	↑	[55,57]
v-ATPase	Maintains PH homeostasis and function of lysosome, binds to Rags and Ragulator	↑	[62]
SLC7A5/SLC3A2	Bidirectional transporter which imports leucine, involved in mTORC1 activation	↑	[45]
SLC1A5	Regulates glutamine uptake, involved in mTORC1 activation	↑	[45]
T1R1/T1R3	G-protein-coupled taste receptor, senses extracellular AAs to promote mTORC1 activation	↑	[93]
TTT-RUVBL1/2	Binds to mTORC1, promotes mTORC1 activation	↑	[84]

best-characterized Rag GTPases (Table 1). However, the discovery of some components significantly conflict with others, obscuring a clear pathway and possibly implicating more than one. In this review, we outline recent advancements made in deciphering the molecular mechanisms involved in the nutrient–mTORC1 signaling cascade, including newly recognized components. Furthermore, we highlight unanswered questions and what future directions should entail.

AMPK sensing of nutrients and energy

AMPK, a serine/threonine kinase, is a crucial cellular energy sensor found in all eukaryotes and is activated when there is an increase in cellular AMP or ADP. Under nutrient starvation conditions, AMPK activation conserves energy for the cell by phosphorylating numerous substrates to inhibit anabolic processes and promote catabolic processes. AMPK is a heterotrimeric complex composed of a catalytic (α) and two regulatory (β and γ) subunits. Myristoylation of the β subunit is required for membrane localization and activation [35]. Low energy status is directly sensed by AMPK; the γ subunit binds to AMP and ADP, enforcing a conformational change that blocks dephosphorylation of AMPK to keep it in an activated state. The phosphorylation of AMPK at threonine 172 in the activation loop is essential for its activation. Liver kinase (LK)B1 (also known as STK11), a tumor suppressor gene mutated in Peutz–Jeghers syndrome, is the major upstream AMPK threonine 172 kinase. LKB1 is allosterically activated through the interaction with the pseudokinase STE20-related adapter (STRAD) and the adaptor protein mouse protein 25 (MO25). Threonine 172 can also be phosphorylated by calcium/calmodulin-activated kinase, CAMKKβ (also known as CAMKK2). However, CAMKKβ-mediated phosphorylation of AMPK occurs with an intracellular increase in calcium, and not necessarily changes in AMP and ADP levels [36].

Under nutrient starvation conditions, AMPK acts as a so-called 'metabolic checkpoint', relaying messages to mTORC1 through direct phosphorylation of TSC2 and Raptor to inhibit cell growth and preserve energy (Figure 2). AMPK blocks mTORC1 activation and signaling by phosphorylating TSC2 and activating its GAP activity [37]. An increase in the GAP activity of TSC2 decreases $Rheb^{GTP}$ and mTORC1 activation. Phosphorylation of TSC2 by AMPK acts as a primer for the phosphorylation and activation of TSC2 function by glycogen synthase kinase (GSK)3-β. Wnt signaling promotes mTOR activation and cell growth through the inhibition GSK3-β [37]. In addition to mTORC1 inhibition through TSC2, AMPK inhibits mTORC1 activation by direct phosphorylation of Raptor, inducing 14-3-3 and Raptor binding [38].

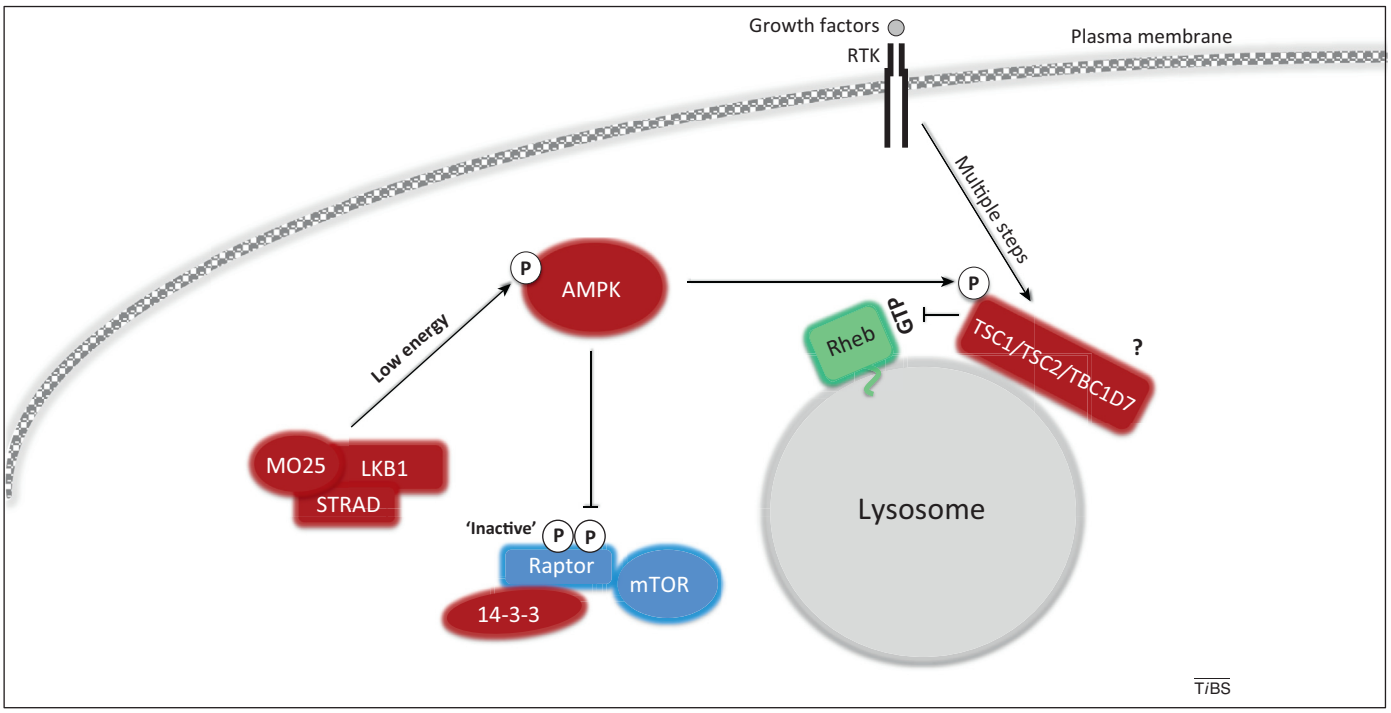


Figure 2. Energy sensing by AMP-activated dependent kinase (AMPK) and control of mammalian target of rapamycin complex 1 (mTORC1). AMPK acts as a metabolic checkpoint under nutrient starvation conditions, translating signals to mTORC1 through direct phosphorylation of tuberous sclerosis complex (TSC)2 and regulatory-associated protein of mTOR (Raptor) and inhibiting cell growth. The phosphorylation of AMPK at threonine 172 by liver kinase (LK)B1 in the activation loop is essential for AMPK activation. LKB1 is in a complex with STE20-related adapter (STRAD) and mouse protein 25 (MO25). AMPK phosphorylates and activates TSC2, which inhibits mTORC1. AMPK also phosphorylates Raptor in a parallel pathway on sites that induce Raptor-14-3-3 binding and inhibition of mTORC1. Abbreviations: P, phosphorylation; RTK, receptor tyrosine kinase.

Hypoxia promotes AMPK–TSC activation and suppresses mTORC1 by reducing ATP production. Also, increased expression of the hypoxia-inducible regulated in development and DNA damage responses 1 (REDD1) gene can inhibit mTORC1 in a TSC-dependent manner [39,40]. Many well worked out nutrient-sensing mechanisms by AMPK in controlling mTORC1 have been documented. It will be interesting what future findings will uncover, especially what additional crosstalk and cues regulate AMPK, which could impact mTORC1 and cell growth.

Amino acid signaling to mTORC1

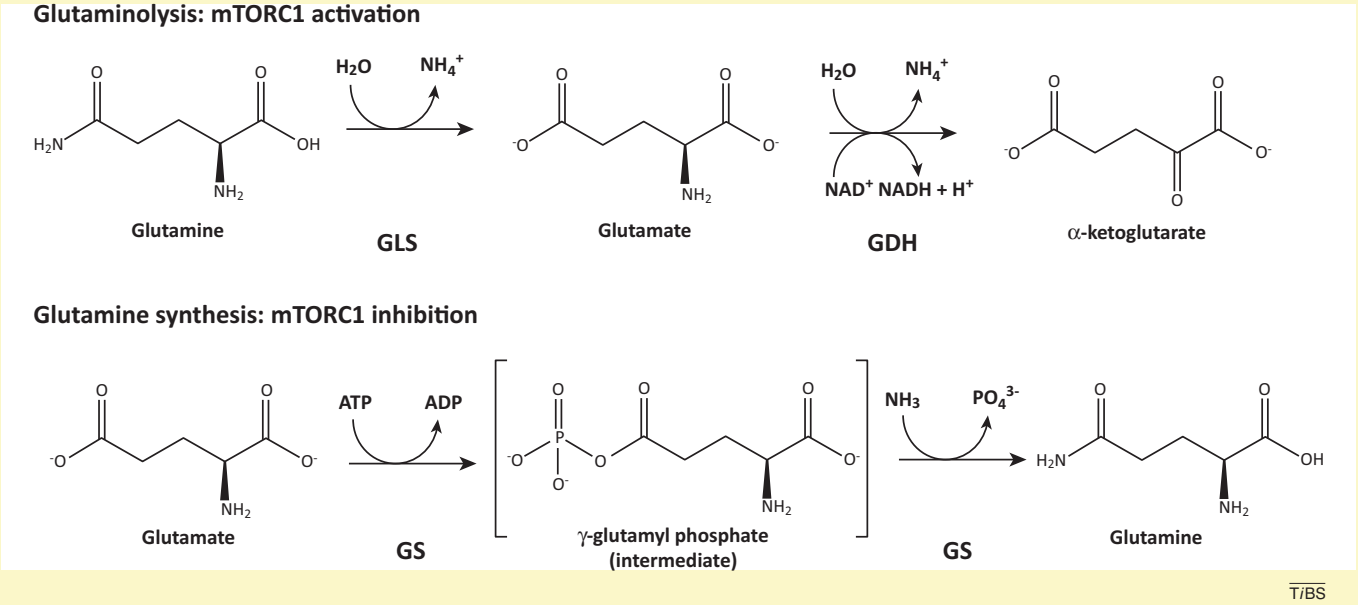
Compared to glucose and energy sensing by AMPK, how AAs are sensed and regulate mTORC1 is poorly understood. AAs are building blocks of proteins that promote cell growth, and not surprisingly, AAs drive the mTORC1 pathway. Organisms tightly coordinate the balance between anabolic and catabolic events to utilize best not only energy but also AAs. In fact, AAs are essential for mTORC1 activation, because growth factors and other stimuli cannot efficiently activate mTOR when AAs are limiting [41–43]. Specific AAs required for the activation of mTORC1 are not completely understood, although withdrawal of the essential AAs leucine and arginine is as efficient as total AA removal in downregulating mTORC1 signaling [44]. Additionally, glutamine is required for extracellular leucine to activate mTOR [45], and recently, glutamine metabolism has been shown to control mTORC1 (Box 1) [46,47]. Exactly how AAs control mTORC1 is currently a hot topic, resulting in many recent publications identifying new components aiming to decode the mechanisms involved in this signaling cascade (Table 1).

Rag GTPases

Center stage in the AA-dependent induction of mTORC1 is the Rag family of GTPases, possibly the strongest link to date between AA signaling and mTORC1 (Figure 3). Two independent groups have identified this connection using different approaches. One study in mammalian cells identified the Rags as Raptor-interacting proteins through an immunoprecipitation–mass spectrometric analysis approach, and the other screened small GTPases using RNAi in *Drosophila* cells [41,48]. There are four Rag proteins in mammals: RagA and RagB (~98% sequence similarity) and RagC and RagD (~87% sequence similarity) [12]. RagA or RagB forms a heterodimer with RagC or RagD; thus, four different complexes are possible. Ras family GTPases have not been reported to form stable dimers, making this an exclusive feature of the Rags. In yeast, the Rag proteins are homologs of Gtr1 (RagA or RagB) and Gtr2 (RagC or RagD). The first connection between the Gtr1-Gtr2 complex and TORC1 in yeast was the discovery of Gtr2 as a potential downstream effector of TORC1 [49]. Structurally, both Gtr1 and Gtr2 are composed of an N-terminal Ras-like GTPase domain important in guanine nucleotide binding and a C-terminal domain that is crucial for Gtr1–Gtr2 interaction [50]. AAs promote the active conformation of the Rag complex, in which RagA/B is loaded with GTP ($RagA/B^{GTP}$) and RagC/D is loaded with GDP ($RagC/D^{GDP}$). Under AA-rich conditions the $RagA/B^{GTP}$–$RagC/D^{GDP}$ heterodimer physically interacts with Raptor to recruit mTORC1 from an undefined location within the cell to the lysosome. Immunofluorescent studies show that withdrawal of AAs results in the dispersion of endogenous mTORC1 throughout the cell, whereas AA

Box 1. Regulation of mTORC1 by glutamine metabolism

Glutamine is the most abundant AA in the blood. The metabolism of glutamine occurs through a process of double deamination called glutaminolysis, to produce α-ketoglutarate (KG) (Figure I). First, glutamine is deaminated by glutaminase (GLS) to produce glutamate. Conversion of glutamate to αKG is performed by glutamate dehydrogenase (GDH). Leucine is an allosteric regulator of GDH, directly binding to GDH, aiding in the promotion of glutaminolysis by stimulating deamination of glutamate to form αKG. Glutaminolysis has recently been implicated to stimulate Rag–mTORC1 signaling. αKG is thought to activate mTORC1 through a family of αKG-dependent dioxygenases, prolyl hydroxylases. Glutamine metabolism is important to sustain ATP levels, through αKG replenishing the tricarboxylic acid (TCA) cycle, and cancer cells are thought to be addicted to glutamine. Glutamine in combination with leucine increases RagA/B^{GTP} promoting mTORC1 activation (see Figure 3 in main text). Chemical inhibition of glutaminolysis by 6-diazo-5-oxo-L-norleucine (DON) decreases RagA/B^{GTP} formation, mTOR lysosomal localization, and thus, mTORC1 activation. Consistently, overexpression of the constitutively active Rag complex, RagA/B^{GTP}–RagC/D^{GDP}, reverses mTORC1 inhibition by DON. Furthermore, glutaminolysis has been demonstrated to regulate autophagy and cell size through mTORC1 [46]. Consistently, elevation of glutamine synthetase (GS), the enzyme that catalyzes the opposite reaction to that of GLS, inhibits mTORC1 lysosomal translocation and activation, and promotes autophagy [94]. These findings demonstrate the crosstalk between metabolism and cell signaling and begin to build a model in how mTORC1 may sense the fluctuations of glutamine and leucine together. αKG production from glutamine activates mTORC1 and inhibits autophagy, whereas the synthesis of glutamine inhibits mTORC1 and promotes autophagy.



TiBS

Figure I. Mammalian target of rapamycin complex 1 (mTORC1) activation controlled by glutamine. Glutamine metabolism controls mTORC1. Top – Glutaminolysis, the metabolism of glutamine to α-ketoglutarate (KG), is a two-step process that results in the activation of mTORC1. Glutamine is first deaminated by glutaminase (GLS) to produce glutamate, and then glutamate dehydrogenase (GDH) produces αKG. Bottom – By contrast, the synthesis of glutamine inhibits mTORC1 activation. Glutamine is synthesized by glutamine synthetase from glutamate.

stimulation promotes the colocalization of mTORC1 and lysosomal-associated membrane protein (LAMP)2. The Rags are thought to bind to mTORC1 at the lysosome, placing it in close proximity to Rheb, a potent activator of mTORC1. A mutated constitutively active Rag complex (RagA/B^{GTP}–RagC/D^{GDP}) can bind to and activate mTORC1 in the absence of AAs. Consistently, the inactive Rag complex (RagA/B^{GDP}–RagC/D^{GTP}) does not interact with mTORC1 and suppresses mTORC1 activity even in the presence of AAs [41,51]. Therefore, the guanine nucleotide status of the Rag complex is crucial in modulating the activation of mTORC1. The identification of Rag guanine nucleotide exchange factors (GEFs) and GAPs will be significant in understanding AA signaling to mTORC1 (Box 2).

Rheb is required for AA-induced mTORC1 activation, as demonstrated by the observation that AAs fail to activate mTORC1 in Rheb knockout cells [42,52]. Notably, Rheb may not be directly involved in AA signaling to mTORC1. Fluorescent microscopy studies using overexpressed tagged Rheb have suggested that it resides at the lysosome, but no efficient endogenous antibody is currently available to confirm this result. Recently, additional localization studies using an efficient endogenous TSC2 antibody have shown that TSC2 is also localized at the lysosome in further support of Rheb residing there [33]. As previously mentioned, growth factors such as insulin signal through the TSC pathway to increase Rheb^{GTP} and promote mTORC1 activation, whereas AAs translocate mTORC1 to lysosome via the Rags. This is an attractive model because it explains how mTORC1 may combine multiple inputs, such as growth factors and AAs, in controlling cell growth. Further supporting this model, it has been reported that AAs promote Rheb–mTOR binding [52,53]. Rheb overexpression can activate mTORC1 even under AA starvation. The overexpressed Rheb may be localized throughout the cell [51,54]; therefore, it is available to activate mTORC1 even when mTORC1 is not localized on lysosomes. Consistent with this model, artificially targeting Raptor to the lysosome surface activates mTORC1 regardless of the AA status [51].

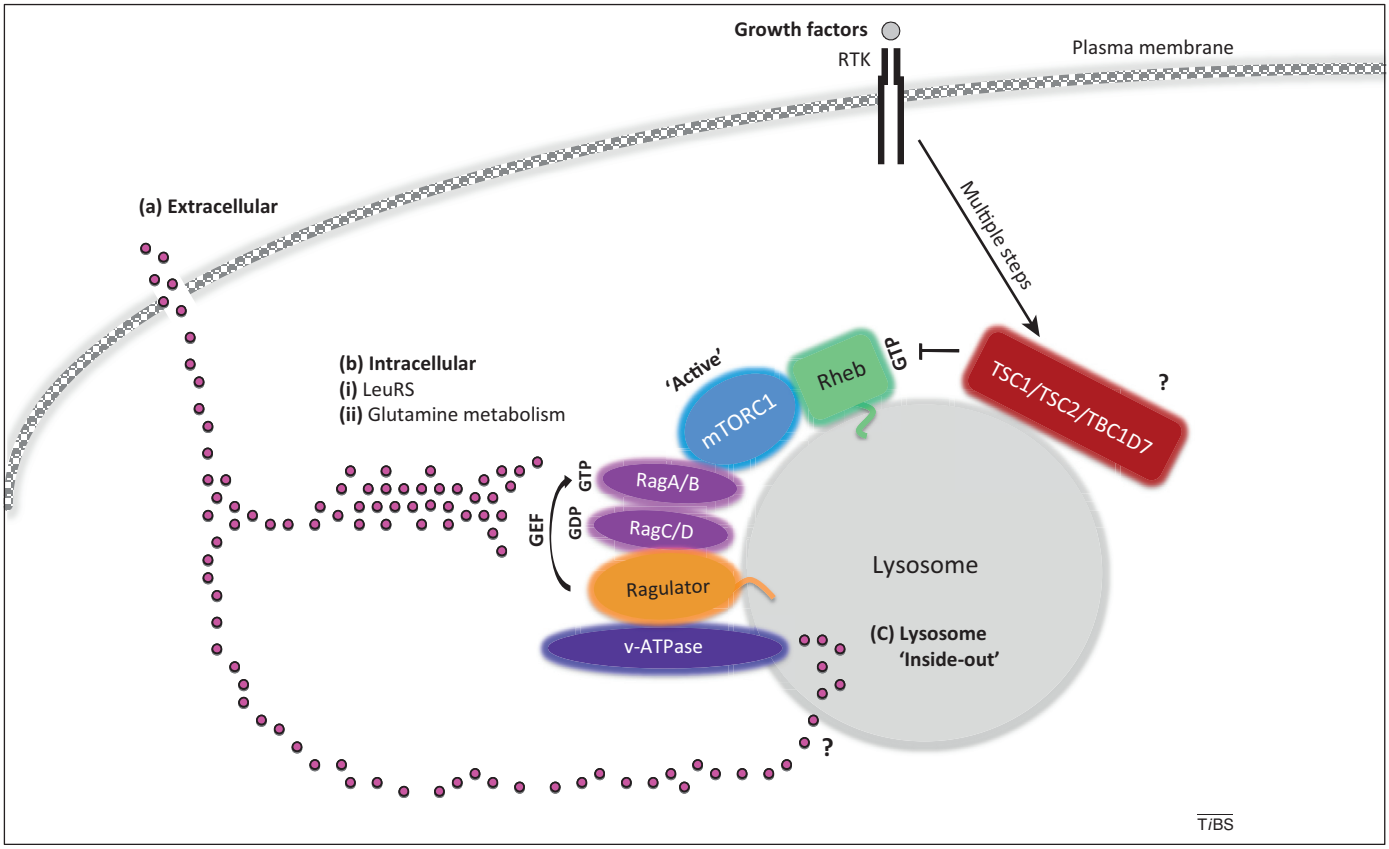


Figure 3. Amino acid (AA) signaling to mammalian target of rapamycin complex 1 (mTORC1). AAs, depicted as pink circles, may be sensed by mTORC1 **(a)** extracellularly, **(b)** intracellularly, from within the **(c)** lysosome, or possibly through a combination. In addition, the specific AA sensed to promote mTORC1 activation is still not clear. Although, leucine, glutamine, and arginine appear to be essential in activating mTORC1. It has been documented that mTORC1 can sense (i) leucine through leucyl-tRNA synthetase (LeuRS) and (ii) glutamine levels. Glutaminolysis results in mTORC1 activation, whereas glutamine synthesis seems to inhibit mTORC1 (Box 1). In response to AAs, an active RagA/B^{GTP}–RagC/D^{GDP} physically interacts with regulatory associated protein of mTOR (Raptor) and recruits mTORC1 to the lysosome to promote its activation by Rheb, another GTPase. Recently, tuberous sclerosis complex (TSC) has also been reported to localize at the lysosome (TSC1 and Tre2-Bub2-cdc16 (TBC)1 domain family, member 7 (TBC1D7) have not yet been confirmed to reside at the lysosome, denoted by?). The mTORC1 interaction with Rag A/B^{GTP}–Rag C/D^{GDP} is anchored to the lysosome by a complex called the Ragulator. The Ragulator also serves as a guanine exchange factor (GEF) for Rag A/B^{GTP}, promoting mTORC1 activation. Moreover, the vacuolar H+-ATPase (v-ATPase) has been observed to be upstream of the Ragulator in AA sensing to mTORC1. The v-ATPase senses AA from within the lysosomal lumen, through an 'inside-out' mechanism. How AAs accumulate inside the lysosome and what transporters are involved in this process is still unknown, denoted by '?'. Growth factors, through the TSC pathway, activate Rheb (Rheb^{GTP}) so that it can turn on the kinase activity of mTORC1, whereas AAs through the Rags localize mTORC1 in close proximity to Rheb at the lysosome. Abbreviation: RTK, receptor tyrosine kinase.

Amino acid sensing at the lysosome

The Rag proteins lack membrane-targeting sequences, unlike other typical small GTPases such as Rheb. Thus, the Rag–mTORC1 complex is anchored to the lysosome surface by a complex called the 'Ragulator'. The Ragulator was originally characterized as a trimeric complex composed of three small proteins: p18, p14, and MAPK scaffold protein 1 (MP1), which are encoded by the late endosomal/lysosomal adaptor and MAPK and mTOR activator 1, 2, and 3 (LAMTOR1, LAMTOR2, and LAMTOR3) genes, respectively. Recently, two additional members have been added to the Ragulator complex, C7orf59 and hepatitis B virus X interacting protein (HBXIP) (suggested to be renamed LAMTOR4 and LAMTOR5, respectively, for consistency), resulting in a pentameric Ragulator complex. Significantly, the pentameric Ragulator complex has GEF activity, but not the individual subunits or the trimeric Ragulator towards RagA/B (Box 2) [55]. Rag binding to the Ragulator holds mTORC1 at the lysosome due to p18 N-terminal myristoylation and palmitoylation [51]. Components of the Ragulator complex have also been documented to act as an anchor element for mitrogen-activated protein kinase (MEK) in the MEK/extracellular signal-regulated kinase (ERK) pathway, although the functional relationship to mTORC1 activation is unclear [56]. Depletion of the Ragulator components disrupts Rag localization to the lysosome and mTORC1 activation. It is currently unclear if the Rags are always lysosomally localized, or if they relocalize in response to particular conditions, such as AA stimulation. In yeast, there are no obvious homologs of the Ragulator components; but the exit from growth arrest (EGO) complex that is also localized to the vacuole (lysosome equivalent) similarly regulates Gtr1–Gtr2 [49,57]. Although the Ragulator components are not conserved, the high-resolution crystal structure of Ego3 is strikingly similar to that of MP1 and p14, suggesting an overall structural conservation [58]. Interestingly, the structures of MP1 and p14 contain a region called a roadblock domain, which is also predicted to be present in RagA/B and RagC/D [50,59,60]. Understanding the function and significance of this domain may be of great interest in better understanding AA signaling to mTORC1 and identifying additional components implicated in this pathway.

The precise location of where AAs originate from and are sensed by mTORC1 is still unclear. Recent findings suggest that AA sensing may begin within the lysosome in an

Box 2. GEFs and GAPs involved in amino acid signaling to mTORC1

An important piece in AA signaling to mTORC1 and cell growth is the identification of the Rag GEFs and the opposing GAPs. GEFs usually activate GTPases by promoting the exchange of GDP to GTP, whereas GAPs act antagonistically to deactivate GTPases by increasing their intrinsic rate of GTP hydrolysis [95]. RagA/B^{GTP} heterodimerizes with RagC/D^{GDP} in order to be active. Therefore, orchestration of the GEFs and GAPs for the Rags and other small GTPases that are involved in signaling to mTORC1 is key for mTORC1 activation. A new study, identifying the lysosomal localized RagA/B GEF as the Ragulator, has just emerged. The Ragulator is now classified as a pentamer, with the identification of the two new components, C7orf59 and HBXIP (*LAMTOR4* and *LAMTOR5*, respectively). All five components of the Ragulator complex appear to be required for GEF activity towards RagA/B [55]. Vacuolar morphogenesis 6 (Vam6) has been reported to function as a Gtr1 (RagA/B homolog) GEF in *Saccharomyces cerevisiae* [57]. However, the mammalian vacuolar protein sorting factor 39 (VPS39) homolog of Vam6 has no GEF activity towards RagA/B [55]. Two studies have identified LeuRS as a component playing a role in mTORC1 activation by AAs. Interestingly, LeuRS has been shown both in yeast and mammalian cells to be involved in AA signaling to mTORC1, although the proposed mechanisms are rather different between yeast and mammals. In yeast, LeuRS directly interacts with Gtr1^{GTP}, inhibiting an unknown GAP and keeping TORC1 active [72]. In mammals, LeuRS binds to and acts as a GAP for RagD (a Gtr2 homolog) in the cytoplasm. LeuRS facilitates an active RagD form by promoting RagD^{GDP} [71]. Missing in the current model is the RagA/B GAP and Rheb GEF. Future studies identifying GEFs and GAPs and how they are regulated will provide critical new insights into AA sensing and cell growth control.

'inside-out' type of signaling mechanism. Autophagy generates an AA supply for the cell through degradation of autophagosome contents within lysosomes under nutrient starvation conditions [12]. Inhibition of mTORC1 is required for the initiation of autophagy, but complete mTORC1 inhibition blocks termination of autophagy [61]. Moreover, mTORC1 is reactivated after prolonged starvation conditions in an autophagy-dependent manner, possibly due to the generation of AAs by autophagy. These observations suggest that some type of intricate relation and balance between AAs, mTOR, and autophagy is needed to maintain activation of mTORC1 and cell growth [21,61]. It will be intriguing to understand if the generation of AAs by autophagy controls mTORC1, and under what conditions.

Sabatini and colleagues have reported a model in which AAs accumulate inside the lysosomal lumen and are eventually detected by the large multisubunit vacuolar H^+-ATPase (v-ATPase), which in turn signals to the Ragulator–Rag complex via direct interaction, hence, the inside-out mechanism [62]. The v-ATPase, which consists of V1 and V0 domains, is essential for maintaining the low pH necessary for the lysosome to function properly. The V1 domain hydrolyses ATP, rotating the V0 membrane domain to pump protons across the plasma membrane and into the lysosomal lumen, acidifying it. Depletion of the v-ATPase subunits inhibits mTORC1 localization and activation. Supporting this, treatment of cells with two different v-ATPase inhibitors, concanamycin A and salicylihalamide A, halts mTOR localization to the lysosome and activation in response to AAs [62]. Although many components have been shown to be involved in AA mTORC1 activation at the lysosome, the precise sensor of AAs is still not known. Furthermore, the source of the build-up of AAs inside the lysosome is unclear. Whether this lysosomal pool of AAs is the end result of autophagy, shuttled in from outside of the lysosome (extracellularly or intracellularly), or a combination of the two, needs further investigation.

Many AA transporters have been identified at the lysosome and some have been documented to modulate mTORC1 activity [63–65]. In addition, a study has demonstrated through the radiolabeling of AAs that extracellular AAs can accumulate within and be shuttled out of the lysosome [62]. One such transporter identified is the proton-assisted solute carrier family 36 (SLC36) AA transporters (PATs), which have a potent effect on mTORC1-mediated growth [65]. This mechanism may be conserved: *Drosophila* CG3424 and CG1139, two PAT-like transporters, control growth [66]; in mammals PAT1 is required for mTORC1 activation and cellular proliferation [65]. Instead of transporting AAs into the lysosome, PAT1 exports AAs from the lysosomal lumen to the cytosol [63]. One model has proposed that AAs promote the formation of a complex, termed the 'nutrisome' or AA-sensing engine, comprising PAT1, Rags, Ragulator, and v-ATPase, that altogether are required to activate mTORC1. In this model, growth-factor-stimulated activation promotes the shuttling of PAT1 from the cell surface to the endosome/lysosome, where PAT1 is able to form the nutrisome. The v-ATPase pumps protons into the lysosome, cycling protons for PAT1 to transport the AAs out of the lysosome, and somehow activating mTORC1 in the process [67]. Alternatively, another group has found that the overexpression of PAT1 completely suppresses AA-dependent mTORC1 activation by depleting the AA pool from within the lysosome. Inhibition is relieved by overexpressing RagA/B^{GTP}, which is as efficient as the RagA/B^{GTP}–RagC/D^{GDP} heterodimer in rescuing mTORC1 inhibition under AA starvation conditions. This model implies that maintaining the AA pool inside the lysosome is important for the activation of mTORC1, and that PAT1 decreases mTORC1 activation by facilitating the export of AAs out of the lysosome [62]. Also worth noting, PAT1 is specific for transporting alanine, glycine, and proline. This somewhat complicates the picture due to several studies implicating leucine, arginine, and glutamine, as the main activators of mTORC1 [68].

Small GTPases associated with vesicle transport or lysosomes have been documented to play a role in AA mTORC1 activation. Besides Rheb and the Rags, ras-like protein A (RalA), Rab protein 5 (Rab5), and ADP-ribosylation factor 1(Arf1) have been implicated in this pathway. Depletion of RalA and its activator Ral guanine nucleotide stimulator (GDS) antagonizes the ability of AAs and glucose, but not insulin, to signal to mTORC1 [69]. AA availability regulates the amount of GTP-bound RalA and thus its activation; active RalA partially rescues mTORC1 activation in the absence of Rheb. Although this is a promising finding, the question of whether or not active RalA can rescue mTORC1 in AA depletion studies has yet to be tested. Rab and Arf, which are key mediators in vesicle transport, have been identified as mTORC1 inhibitors

through knockdown studies on small GTPases in *Drosophila*. Interestingly, activated Rab5 and Arf1 also block AA-induced mTORC1 signaling in mammalian cells, whereas glucose-stimulated mTORC1 signaling is unaffected. Furthermore, active Rab5 selectively inhibits mTORC1 through the Rags but not Rheb [70]. These data may indicate that intracellular vesicle movement is important for AA-induced mTORC1 activation. Whether or not additional small GTPases play a role in AA signaling to mTORC1 has yet to be determined.

Other components implicated in amino acid signaling to mTORC1

Leucyl-tRNA synthetase

Two recent studies have shown that leucyl-tRNA synthetase (LeuRS), the enzyme that charges leucine to its cognate tRNA, also functions as a leucine sensor in the activation of mTORC1 [71,72]. Leucine appears to be crucial for AA-dependent mTORC1 activation in most cells [44]. Interestingly, this mechanism has been shown in both yeast and mammalian cells, although the details differ greatly. In yeast, LeuRS binds to $Gtr1^{GTP}$ (the homolog in mammalian $RagA/B^{GTP}$), preventing GTP hydrolysis and locking Gtr1 in its active form, which signals to TORC1 [72]. In mammals, LeuRS directly interacts with and functions as a GAP for RagD, but not RagC, promoting its activation in a leucine-dependent manner. Unexpectedly, LeuRS binds to the C-terminal domain of RagD, which has been shown to be crucial for Gtr1–Gtr2 binding and thus TORC1 activation in yeast [71]. Notably, the arginine residue in human LeuRS that is essential for its GAP activity is not conserved in the *Drosophila* LeuRS homolog. This is rather surprising because activation of mTORC1 by AAs is conserved in all eukaryotes, including *Drosophila*. LeuRS sensing occurs in the cytoplasm and not at the lysosome, possibly implicating multiple AA sensing pathways controlling mTORC1 (Figure 3). Further work is needed to elucidate the precise mechanism or role of LeuRS in translating AA levels into mTORC1 activation. Multiple other components have been reported to sense AAs not only at the lysosome, but also in the cytoplasm and at the plasma membrane, and incorporating them all into a simplified model is going to be a very difficult task (Table 1).

mLST8 and inositol polyphosphate multikinase (IPMK): components of the nutrient-sensitive mTOR–Raptor complex

Nutrients can regulate mTOR–Raptor binding. In nutrient deprivation, the Raptor–mTOR interaction is slightly tighter, which somehow inhibits the kinase activity of mTOR but is lost with the addition of AAs [73]. mLST8 (GβL) is required for both the nutrient- and rapamycin-dependent interaction between Raptor and mTOR, possibly implicating its involvement in AA sensing [74]. In addition to mLST8, IPMK has been shown to regulate the nutrient-sensitive mTOR–Raptor complex. IPMK controls the mTOR–Raptor interaction in response to AAs, a relation that appears to be independent of IPMK catalytic activity. Disruption of the mTOR and Raptor interaction through IMPK depletion correlates with a significantly weakened association between mTOR and the active Rag complex, although Raptor–Rag binding remains unaffected. Like mLST8, nutrient deprivation enhances Raptor, mTOR, and IPMK binding, which is reversed by the addition of AA. These results suggest that mTOR–Raptor binding may be nutrient sensitive, possibly through mLST8 and IPMK [75].

General control non-repressed (GCN)2

GCN2 is a protein kinase that monitors nutrient availability by binding to uncharged tRNA. Increased GCN2 activity can increase the expression of genes involved in AA biosynthesis and transport through activating transcription factor 4 (ATF4) [76]. One possible pathway for the AA GCN2 mechanism is through the protein phosphatase 1 regulatory protein growth arrest and DNA damage-inducible protein (GADD34), which is upregulated by ATF4 and binds to TSC1/2, promoting the dephosphorylation of a crucial AKT site on TSC2. This causes an increase in TSC2 GAP activity, a decrease in $Rheb^{GTP}$, and inhibition of mTORC1 [77,78]. However, this transcription-dependent mechanism is at odds with the rapid activation of mTORC1 by AA, which causes S6K phosphorylation to increase within minutes. Interestingly, GCN2 knockout mice have impaired phosphorylation of mTORC1 substrates (4EBP1 and S6K1) and demonstrate an increased lethality when they are deprived of leucine [79,80]. Understanding the role of GCN2 in AA signaling to mTORC1 will be interesting.

Phospholipase (PL)D

Another study has shown that Rheb binds to PLD1, an upstream serum-activated positive regulator of mTORC1, in a Rheb bound GTP-dependent manner, and thus stimulates the activity of mTORC1 *in vitro*. AA starvation inhibits PLD activation in response to serum, linking PLD–Rheb to the AA induction of mTORC1. The class III PI3K, related to yeast Vacuolar Protein Sorting factor 34 (VPS34), which phosphorylates phosphatidylinositol at the 3′-OH to form phosphatidylinositol 3-phosphate (PI3P), has also been implicated as a bridge between AA signals and mTORC1. AA-induced VPS34 activation has been shown to promote PLD localization to the lysosome, in close proximately to Rheb, through production of PI3P. PI3P binds numerous proteins containing PI3P-targeting phox homology (PX) domains, including PLD [81,82].

Src homology 3 domain binding protein (SH3BP)4

A negative regulator of AA-induced mTORC1 activation is SH3BP4, which is often deleted in breast and renal cancers. It inhibits AA signaling to mTORC1 in leucine-deprived conditions by binding to the inactive Rag complex via its SH3 domain, preventing the formation of the active Rag complex. This results in decreased Raptor binding and shuttling of mTORC1 to the lysosome. Consistently, knockdown of SH3BP4 in leucine-stimulated conditions improves mTORC1 activation, increasing cell proliferation and size [83].

TTT–RUVBL1/2 complex

The Tel2–Tti1–Tti2 (TTT)–RuvB–like 1 and 2 (RUVBL1/2) complex has been identified as a strong regulator of mTORC1 in $TSC2^{-/-}$ cells, indicating that it regulates mTORC1 independently of growth factors [84]. TTT–RUVBL1/2 consists of the components telomere maintenance 2 (Tel2), and Tel2

interacting proteins 1 and 2 (Ttil1 and Ttil2), which were previously identified to regulate the assembly of PIKK-containing complexes such as mTOR [85–87]. Additionally, the TTT–RUVBL1/2 complex also contains the ATPases RUVBL1/2, and other components such as RNA polymerase II associated protein 3 (RPAP3), PIH1 domain containing 1 protein (PIH1D1), and heat shock protein (Hsp)90. Interestingly, TTT–RUVBL1/2 complex mRNAs appear to be elevated in cancer tissues. Energy stress disassembles the ATP-dependent TTT–RUVBL1/2 complex, which in turn results in a decreased TTT–RUVBL1/2–mTORC1 interaction. Loss of TTT–RUVBL1/2 results in the mislocalization of mTORC1 to the lysosome, mTORC1 dimerization, and a decrease in mTORC1–Rag interaction [84].

Concluding remarks

The sensing of nutrients, growth factors, and stress all converge on the master regulator mTORC1 in order to control cell growth and physiology appropriately. Although many of the signaling cascades from growth factors and stress to mTORC1 are well characterized, the pathways that lead from nutrients to mTORC1 activation are less clear. Much progress has been made in understanding energy sensing by AMPK to mTORC1, however, significant work is still needed in the AA-sensing pathway. Numerous components have been identified and characterized to function in this signaling cascade and multiple pathways may be involved. The Rag GTPases have emerged as a key molecular switch downstream of AAs to modulate mTORC1 activity. Clarifying which AAs are critical and where they are originating from (extracellularly, intracellularly, or from within the lysosome) in mTORC1 activation would be beneficial in identifying sensors. Connecting all of the current known components to precise mechanisms, and to one another, is going to be a major challenge in completely understanding how mTORC1 senses AAs.

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Amino acid signalling upstream of mTOR

Jenna L. Jewell, Ryan C. Russell and Kun-Liang Guan

Abstract | Mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase that is part of mTOR complex 1 (mTORC1), a master regulator that couples amino acid availability to cell growth and autophagy. Multiple cues modulate mTORC1 activity, such as growth factors, stress, energy status and amino acids. Although amino acids are key environmental stimuli, exactly how they are sensed and how they activate mTORC1 is not fully understood. Recently, a model has emerged whereby mTORC1 activation occurs at the lysosome and is mediated through an amino acid sensing cascade involving RAG GTPases, Ragulator and vacuolar H^+-ATPase (v-ATPase).

Cells and organisms need to integrate information from the environment to ensure that they only grow when conditions are favourable. The highly conserved Ser/Thr protein kinase target of rapamycin (TOR) is a key integrator of environmental cues, including nutrient and growth factor availability as well as stress. Under nutrient-rich conditions, TOR promotes cell growth by stimulating biosynthetic pathways, including protein synthesis, and by inhibiting cellular catabolism such as through repression of the autophagy pathway (BOX 1). Therefore, understanding how the different stimuli are detected and how they signal to TOR is integral to elucidating how the cell or organism grows.

Mammalian TOR (mTOR) is part of a complex known as mTORC1, comprising: regulatory-associated protein of mTOR (RAPTOR), which aids in substrate recognition; 40 kDa Pro-rich AKT substrate (PRAS40; also known as AKT1S1) and DEP domain-containing mTOR-interacting protein (DEPTOR), both of which are negative regulators of mTORC1; and mammalian lethal with SEC13 protein 8 (mLST8; also known as GβL), which positively regulates mTORC1. Some mTORC1-activating stimuli, such as growth factors, signal through the tuberous sclerosis complex (TSC; comprising TSC1 and TSC2) (FIG. 1). TSC is a GTPase-activating protein (GAP) for the small GTPase RHEB (RAS homologue enriched in brain) and negatively regulates mTORC1 by promoting RHEB·GTP hydrolysis, converting RHEB into its inactive GDP-bound state. As a result, inhibition of TSC by growth factors gives rise to GTP-bound RHEB, which is a potent activator of mTORC1 kinase activity (reviewed in REFS 1,2). mTORC1 is inhibited directly and indirectly through AMP-activated protein kinase (AMPK)-mediated phosphorylation of RAPTOR and TSC2, respectively, in response to low energy[3,4].

Unlike the other stimuli, amino acids do not signal through TSC–RHEB, and it is currently unclear how amino acid sufficiency or limitation is sensed to modulate mTORC1 activity. It seems that amino acids are the most crucial signals for mTORC1 activation, as growth factors cannot efficiently activate mTOR when amino acids are limiting[5–7]. Although Leu[5,6], Gln and Arg[6,8–12] have been implicated in mTORC1 activation, which specific amino acids are sensed to activate mTORC1 is currently uncertain. Moreover, it remains elusive where the amino acids are first detected, but multiple studies have recently been published that implicate components residing at the lysosome surface.

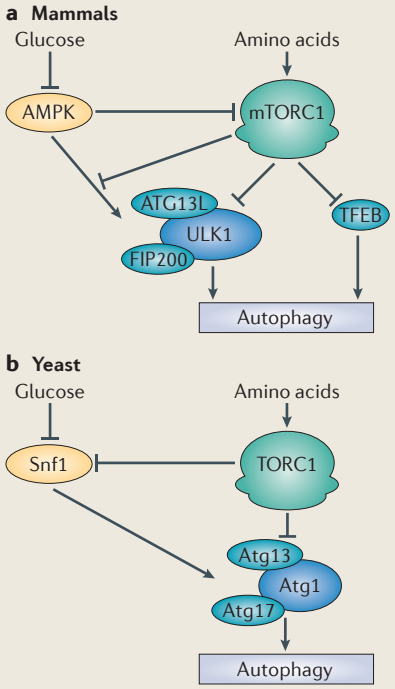
In this Progress article, we summarize the current understanding of the protein cascade that senses amino acids and leads to mTORC1 activation at the lysosome, ultimately resulting in cell growth or inhibition of autophagy. In addition, we highlight what outstanding questions remain to be addressed.

Amino acid sensing at the lysosome

RAG GTPases. RHEB is required for mTORC1 activation by all stimuli, including amino acids. However, *Tsc2*-null mouse embryonic fibroblasts (MEFs) are still sensitive to amino acid starvation, indicating that other components that are not part of the TSC–RHEB pathway are likely to be involved in this process[13,14] (FIG. 1). The discovery of the small RAG GTPases, through screening in *Drosophila melanogaster* and in mammalian cells, was an important breakthrough in understanding amino acid signalling to mTORC1 (REFS 5,15). RAG GTPases belong to the RAS superfamily; however, they have unique characteristics that distinguish them from other small GTPases, such as a long carboxyl-terminal domain, the lack of a membrane-targeting sequence and the ability to form heterodimers[16,17]. In mammals, there are four RAG proteins: RAGA and RAGB (Gtr1 in yeast[52]), which have high sequence similarity and are functionally redundant; and RAGC and RAGD (Gtr2 in yeast[53]), which are also highly related in sequence and are functionally equivalent. RAGA or RAGB forms a heterodimer with RAGC or RAGD, with the possibility of forming four distinct complex arrangements[17]. The crystal structure of the yeast Gtr1–Gtr2 heterodimer was recently solved. The structure reveals an amino-terminal GTPase domain on each protein where GTP binding and hydrolysis occur, and a carboxy-terminal domain on each protein with multiple contacts required for Gtr1·GTP–Gtr2·GDP dimerization[18,19]. Dimerization of RAG GTPases is important for mTORC1 activation and RAG protein stability[5,15].

Like other small GTPases, the activation state of RAG GTPases is reflected by their guanine nucleotide state, and this is regulated by amino acids. Specifically, the presence of amino acids promotes the formation of the active complex configuration, in which RAGA and RAGB are GTP-bound and RAGC and RAGD are GDP-bound (for simplicity, RAGA/B·GTP–RAGC/D·GDP denotes the active complex

Box 1 | **mTORC1 and autophagy**



Macroautophagy, hereafter referred to as autophagy, is the primary catabolic cellular degradation process that generates nutrients and energy to maintain essential cellular activities upon nutrient starvation[42]. Target of rapamycin complex 1 (TORC1) is a potent repressor of autophagy in all eukaryotes[43]. Mammalian TORC1 (mTORC1) is inhibited under starvation conditions by the energy sensor AMP-activated protein kinase (AMPK) and by amino acid signalling. Under these conditions, ULK1 (unc-51-like kinase 1) undergoes autophosphorylation and phosphorylates ATG13L (ATG13-like) and FIP200 (FAK family kinase-interacting protein of 200 kDa), thus forming an active kinase complex to initiate autophagy. Under conditions of nutrient sufficiency, mTORC1 is active and inhibits autophagy by phosphorylating ATG13L and ULK1 subunits, thus repressing ULK1 kinase activity[44–47] (see the figure, part **a**). The ULK1 complex is also activated by AMPK, and this regulation was recently shown to be antagonized by mTORC1-mediated phosphorylation of ULK1 on Ser757 (REF. 44). mTORC1 also indirectly regulates autophagy by controlling lysosome biogenesis through phosphorylation of transcription factor EB (TFEB)[48,49], which drives the transcription of several lysosome- and autophagy-specific genes. mTORC1 and TFEB colocalize to the lysosomal membrane, where mTORC1-mediated phosphorylation promotes TFEB cytoplasmic sequestration[48].

Like mammalian mTORC1, yeast TORC1 inhibits autophagy through the regulation of the Atg1 kinase complex (see the figure, part **b**). Atg1 (the homologue of mammalian ULK1) forms an active complex with Atg13 and Atg17. The presence of amino acids induces TORC1-mediated phosphorylation of Atg13. This phosphorylation event prevents its association with Atg17 and thus blocks autophagy. Inhibition of TORC1 activity by amino acid starvation or rapamycin treatment results in a de-repression of Atg13 and promotion of the active trimeric Atg1–Atg13–Atg17 complex. Another difference between yeast and mammals is that TORC1 inhibits the yeast orthologue of AMPK, termed Snf1. However, it remains unclear whether Snf1 regulates TORC1 (REF. 50). In yeast, Atg1 has been proposed to act downstream of Snf1, although the underlying mechanism remains to be elucidated[51].

Metabolic homeostasis is orchestrated by mTORC1 through the promotion of biosynthetic pathways and the repression of catabolic autophagy in response to energy and amino acid sufficiency. Collectively, the regulation of these processes is strictly determined by energy and amino acid sensing pathways within the cell. Further research, particularly in mammals, is needed to determine whether mTORC1 regulates any aspects of the autophagy machinery in addition to the ULK1 complex. Although it seems that amino acids generated by autophagy have a role in mTORC1 activation, additional studies detailing mechanistic action will be of great importance.

and RAGA/B·GDP–RAGC/D·GTP the inactive complex). Likewise, GTP-bound Gtr1 in complex with GDP-bound Gtr2 is the active form in yeast when amino acids are available (FIG. 2).

Under amino acid-sufficient conditions, the active RAGA/B·GTP–RAGC/D·GDP complex has been shown to bind directly to the mTORC1 component RAPTOR and redistribute mTORC1 to the lysosome. Interestingly, mTORC1 is dispersed throughout the cell under amino acid starvation conditions but is redistributed to vesicles containing lysosome-associated membrane protein 2 (LAMP2) and RAB7 (which are markers of lysosomes and late endosomes, respectively) in response to amino acid stimulation[5,20]. This suggests that RAG GTPases have a role in transducing amino acid signals to mTORC1. Consistent with this, knockdown of RAG GTPases ablates mTORC1 relocalization to the lysosome in response to amino acid stimulation[5]. This relocalization has been proposed to promote the interaction of mTORC1 with RHEB, which is itself thought to be targeted and anchored to the lysosome through its C-terminal CAAX (in which C is Cys, A is an aliphatic residue and X the terminal amino acid) motif[5,20], leading to mTORC1 activation through an unknown mechanism. Although RHEB lysosomal localization has been demonstrated in fluorescent microscopy studies using overexpressed fluorescently tagged RHEB, this has yet to be confirmed for endogenous RHEB owing to the lack of a quality RHEB antibody. A RHEB homologue in budding yeast does not seem to be an upstream activator of TORC1, suggesting divergent evolution of TORC1 regulation[21]. Because RHEB responds to growth factor signalling (FIG. 1), it is possible that mTORC1 regulation by RHEB evolved in organisms in which growth factor signalling occurs.

In contrast to mammals, yeast TORC1 remains at the vacuole, the yeast equivalent of the lysosome, under both starvation and normal conditions. This suggests that the amino acid-induced shuttling mechanism evolved in higher eukaryotes[22] (FIG. 2). However, like the active RAG complex in mammals, Gtr1·GTP–Gtr2·GDP physically binds activated TORC1 in a manner dependent on nucleotide loading and amino acid availability[22].

The nucleotide-bound state of the RAG heterodimer is crucial for mTORC1 activation. Under starvation conditions, overexpression of RAGA/B·GTP alone is sufficient to activate mTORC1, which indicates that it is the nucleotide-bound state of RAGA/B, rather than of RAGC/D, that primarily regulates mTORC1 activation[22,23]. However, co-expression of RAGC/D enhances this effect, in part because RAGC/D stabilizes RAGA/B. Furthermore, an inactive mutant RAGA/B·GDP–RAGC/D·GTP complex that does not respond to amino acids restrains the activity of mTORC1 even under amino acid sufficiency[5,15]. These findings reveal that the presence of amino acids determines the guanine nucleotide state of RAG GTPases and consequently controls the recruitment of mTORC1 to the lysosome and thus its activation. This model suggests a mechanism by which mTORC1 senses multiple stimuli at the lysosome, such as amino acids through RAG GTPases and growth factors through RHEB; amino acids and growth factors seem to converge at the lysosome in order to efficiently activate mTORC1.

Ragulator. RAG GTPases localize at the lysosomal surface but lack a lipid-anchoring motif, which suggests that RAG-binding proteins may tether them to the lysosome. Indeed, mass spectrometry analysis using RAG GTPases as 'bait' identified a complex termed Ragulator that acts as a scaffold for the heterodimeric active RAG complex at the lysosome. Originally, Ragulator was defined

as a trimeric complex consisting of p18 (encoded by *LAMTOR1* (late endosomal/lysosomal adaptor, MAPK and mTOR activator 1)), p14 (encoded by *LAMTOR2*) and MP1 (MEK-binding partner 1; encoded by *LAMTOR3*)[20]. Recently, two additional components, C7orf59 and HBXIP (hepatitis B virus X-interacting protein; encoded by *LAMTOR4* and *LAMTOR5*, respectively) have been described and, together with p18, p14 and MP1, form a pentameric Ragulator complex[24].

But how does Ragulator tether RAG GTPases, and thus mTORC1, to the lysosome? Ragulator and RAG GTPases are tethered at the lysosome by p18, which associates with the membrane via its myristoylated and palmitoylated residues[20,24]. Depletion of Ragulator components disrupts RAG GTPase and mTORC1 lysosomal localization and thus mTORC1 activation, which confirms the importance of this complex in tethering RAG and mTORC1 to the lysosome[20,24].

Ragulator orthologues have not been identified in yeast; however, TORC1 is a component of the Ego complex (EGOC), which is located at the vacuolar membrane (FIG. 2). EGOC consists of Ego1, Ego3, Gtr1 and Gtr2. Although the precise function of Ego3 is not well defined, studies have shown that, like p18, Ego1 is a palmitoylated and myristoylated protein that maintains Gtr1–Gtr2 and TORC1 at the vacuole membrane[25,26]. An important point to keep in mind is that amino acids regulate TORC1 binding to EGOC, similar to mTORC1–RAG–Ragulator binding in mammals. However, unlike in mammals, in which mTORC1 is dispersed across the cell in the absence of amino acids, in yeast TORC1 remains at the vacuole even under starvation conditions. Regardless of the different anchoring machinery, it seems that mTORC1 localization at the lysosome in mammals, or TORC1 at the vacuole in yeast, is crucial in facilitating its function and activation[1,27].

Despite the lack of sequence similarity between EGOC and Ragulator, overall structure conservation is apparent, as Ego3 is structurally similar to MP1 and p14 (REFS 26,28). High-resolution crystal structures of MP1, p14, HBXIP and Gtr1–Gtr2 also identified the presence of a roadblock domain in each protein[29–31]. Structural predictions indicate roadblock domains in the C-terminal domain of RAG GTPases and of C7orf59 (REFS 18,24). Therefore, each component of the RAG–Ragulator complex contains a roadblock domain, six in total, except for the anchoring protein p18, which acts as a scaffold for the roadblock domain-containing proteins. The function of this domain is currently unknown; however, it may provide a specific architectural element for protein–protein interactions during amino acid signalling to mTORC1. It will be interesting to determine whether other proteins that may potentially be involved in amino acid signalling to mTORC1 contain this domain.

Recently, pentameric Ragulator was determined as a guanine exchange factor (GEF) for RAGA/B, promoting the exchange of GDP for GTP and the consequent activation of the RAG complex, which is crucial for mTORC1 lysosomal localization and activation[24]. The identification of Ragulator as the GEF of RAG GTPases provided a key link between amino acid sensing and RAG guanine nucleotide loading (FIG. 3). The entire pentameric Ragulator complex is necessary for GEF activity towards RAGA/B; the originally identified trimeric Ragulator, or each individual Ragulator component on its own, displays no GEF activity towards RAGA/B. Surprisingly, Ragulator does not contain a domain that is homologous to any of the known GEF catalytic domains. Nucleotide-free rather than nucleotide-bound RAGA/B preferentially interacts with Ragulator, a common feature found in other GEF–GTPase interactions[24]. Unlike its effects on RAGA/B, Ragulator does not display any GEF activity towards RAGC/D, perhaps owing to the differences in switch I and switch II regions among the GTPases, which are known to act as recognition motifs for GEF–GTPase interactions. Rapid intrinsic dissociation of GDP from RAGC/D was also demonstrated, possibly suggesting that no GEF is required[24].

As mentioned previously, there are no clear orthologues of the Ragulator components in yeast. Instead, in yeast, Vam6 (a homologue of the mammalian vacuolar protein sorting 39 (VPS39), which promotes late endosome to lysosome fusion) was identified as the GEF for Gtr1 (REFS 22,32). However, VPS39 does not seem to bind to and function as the GEF for RAGA/B in mammals[24]. It is possible that the GEF for Gtr1 in yeast and RAGA in higher eukaryotes has diverged, although this is surprising considering the high degree of sequence similarity between RAGA/B and Gtr1. Mammalian cells express homologues of VPS39, such as TGFβ receptor-associated protein 1 (TGFβRAP1), which have not been assessed as potential GEFs[33]. Therefore, readers should be cautious about a proposed model in which VPS39 functions as a GEF for RAGA/B in mammals until it can be confirmed.

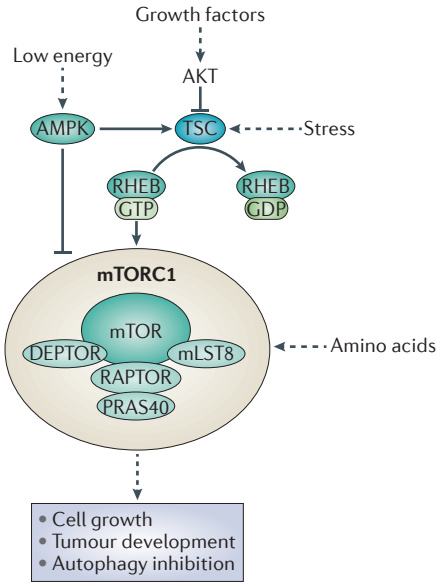


Figure 1 | **The mTORC1 signalling pathway.** Through a multiple-step process, growth factors stimulate AKT, which in turn phosphorylates and inhibits the tuberous sclerosis complex (TSC; comprising TSC1 and TSC2). TSC acts as a GTPase-activating protein (GAP) for the small GTPase RAS homologue enriched in brain (RHEB), promoting hydrolysis of its bound GTP and thus inhibiting RHEB. Upon inhibition of TSC, GTP-bound RHEB levels increase and can potently activate mammalian target of rapamycin complex 1 (mTORC1), presumably at the lysosome. In addition to growth factors, mTORC1 can be regulated by stress, energy status and amino acids. Stress and energy status regulate mTORC1 through TSC. Moreover, AMPK (AMP-activated protein kinase) is activated in response to a low energy status and phosphorylates RAPTOR (regulatory-associated protein of mTOR) and TSC2. This results in the inhibition of mTORC1. Amino acids regulate mTORC1 in a TSC-independent pathway. Collectively multiple stimuli modulate mTORC1 to control cell growth and autophagy. DEPTOR, DEP domain-containing mTOR-interacting protein; mLST8, mammalian lethal with SEC13 protein 8; PRAS40, 40 kDa Pro-rich AKT substrate.

Vacuolar H⁺-ATPase. The findings described above suggest that, in response to the presence of amino acids, Ragulator tethers RAG GTPases to the lysosome, which in turn relocalize mTORC1 to this organelle, leading to mTORC1 activation. What stimulates Ragulator itself in response to changes in amino acid levels? Vacuolar H⁺-ATPase (v-ATPase) interacts with RAG GTPases and Ragulator on the lysosomal membrane, thus regulating mTORC1 activation in response to amino acids both in *D. melanogaster* and mammalian cells[34] (FIG. 3). The highly conserved v-ATPase pumps protons into

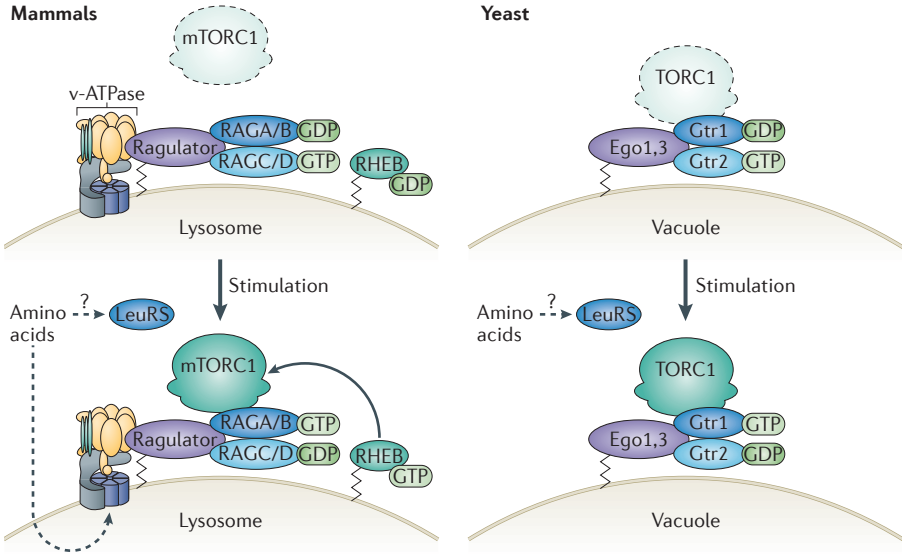


Figure 2 | **Amino acid-induced mTORC1 activation in mammals and yeast.** Amino acids promote the formation of the active configuration of the RAG GTPase complex in mammals at the lysosome (left) and of Gtr1–Gtr2 at the vacuole in yeast (right). Under amino acid starvation conditions in mammals, inactive mammalian target of rapamycin complex 1 (mTORC1) is diffuse throughout the cytosol. The RAG GTPase complex is inactive, with RAGA or RAGB loaded with GDP (RAGA/B•GDP) and RAGC or RAGD loaded with GTP (RAGC/D•GTP) (left, top). During amino acid deficiency in yeast, inactive TORC1 and Gtr1–Gtr2 remain localized at the vacuolar membrane but do not physically interact. Similarly to RAG GTPases in mammals under amino acid-deficient conditions, Gtr1 is bound to GDP and Gtr2 is bound to GTP, which results in an inactive complex (right, top). Amino acid stimulation signals to vacuolar H^+-ATPase (v-ATPase), which is required to induce the guanine exchange factor (GEF) activity of Ragulator (right, bottom). Ragulator acts as a GEF for RAGA/B, promoting the conversion of RAGA/B•GDP to RAGA/B•GTP. Amino acids also facilitate the formation of RAGC/D•GDP, giving rise to the active RAG complex, RAGA/B•GTP–RAGC/D•GDP. The mechanisms involved in switching the guanine nucleotide state of RAGC/D are not clear. mTORC1 binds to the RAG complex and is recruited to the lysosome through an unknown mechanism, where it becomes activated. Leucyl-tRNA synthetase (LeuRS) may act as a direct sensor for the amino acid Leu in the cytoplasm and might be involved in the activation of mTORC1. In yeast under amino acid sufficiency, TORC1, already bound to the vacuole, is activated when Gtr1 is loaded with GTP and Gtr2 is loaded with GDP (left, bottom). LeuRS has also been reported to have a role in TORC1 activation in yeast.

intracellular organelles, such as the lysosome, to acidify them and across the plasma membrane, thereby maintaining cytosolic pH in numerous cell types. v-ATPase is a multicomponent complex composed of two domains: the peripheral cytosolic V_1 domain, which contains eight subunits (subunits A–H); and the integral membrane V_0 domain, which consists of five subunits (subunits a, d, c, c' and c"). The V_1 domain hydrolyses ATP to fuel proton translocation through the V_0 domain channel from the cytoplasm into the lysosomal lumen, resulting in its acidification[35].

The v-ATPase V_1 domain interacts with RAG GTPases, whereas both the V_1 and V_0 domains interact with Ragulator. The interaction of the V_1 domain with RAG GTPases and Ragulator is regulated by amino acids, with the interactions being strengthened by amino acid starvation and weakened in response to amino acid stimulation. Moreover, inhibition of v-ATPase with the macrolide salicylihalamide A renders these interactions unresponsive to amino acids[34]. Similarly to V_1 domain–RAG and V_1 domain–Ragulator interactions, the RAG GTPase–Ragulator interaction also strengthens when cells are deprived of amino acids and weakens with amino acid stimulation[24]. v-ATPase is thought to reside upstream of Ragulator GEF activity. Moreover, chemical inhibition of v-ATPase renders the Ragulator–RAG interaction insensitive to amino acids, implicating Ragulator as a bridge between v-ATPase and RAG in the amino acid cascade[24]. Furthermore, knockdown of individual v-ATPase subunits, or the use of chemical inhibitors, has been shown to not affect RAG localization to the lysosome[34]. It is currently unclear whether RAG GTPases dissociate from the lysosome or whether they are always anchored to this organelle by Ragulator. If RAG GTPases relocalize to another cellular compartment, which signals regulate this movement remains unclear.

What is the role of v-ATPase in amino acid-induced mTORC1 activation? Knockdown of v-ATPase subunits inhibited mTORC1 lysosomal localization and activation, highlighting a key role of v-ATPase in this pathway. Hydrolysis of ATP by v-ATPase is essential for the interaction of RAG GTPases with mTORC1 and consequently for mTORC1 activation, but the reason for this is unknown. v-ATPase-mediated ATP hydrolysis does not seem to drive a proton gradient required for amino acid transport and accumulation within the lysosome, as freely diffusible alcohol ester derivatives of amino acids could still collect within the lysosome regardless of whether the v-ATPase was inhibited. Furthermore, an ionophore that disrupts the lysosomal proton gradient without affecting v-ATPase function had no effect on the RAG GTPase–mTORC1 interaction under amino acid sufficient conditions[34]. Other reports have revealed that a lower cytoplasmic pH correlates with inhibition of mTORC1 activity[36,37], with impaired v-ATPase function increasing cytoplasmic acidification, resulting in mTORC1 inhibition[36,37]. These findings are complimentary to studies showing that chemical inhibition of v-ATPase is important in regulating mTORC1. It thus remains to be determined whether the role of v-ATPase in amino acid signalling to mTORC1 is keeping the lysosome acidic or whether it ensures that the pH within the cytoplasm is optimal for cell growth. Interestingly, in a cell-free system with isolated RAG GTPase-bound lysosomes and purified mTORC1, the addition of amino acids promoted RAG GTPase–mTORC1 binding, indicating that the lysosome contains all of the necessary machinery that is required for amino acid-induced mTORC1 activation[34].

Although the precise amino acid sensor that stimulates v-ATPase is unknown, a recent model has proposed that amino acid signalling to mTORC1 begins within the lysosomal lumen communicating to v-ATPase through an 'inside–out' mechanism[34] (that is, the build-up of amino acids inside the lysosomal lumen signals to and activates mTORC1 that resides outside of lysosomes). Stimulation of starved cells with radiocarbon (^{14}C)-labelled amino acids leads to the rapid accumulation of these amino acids in isolated lysosomes[34]. How the amino acids are transported into the lysosome and what particular transporters facilitate this process is currently unknown.

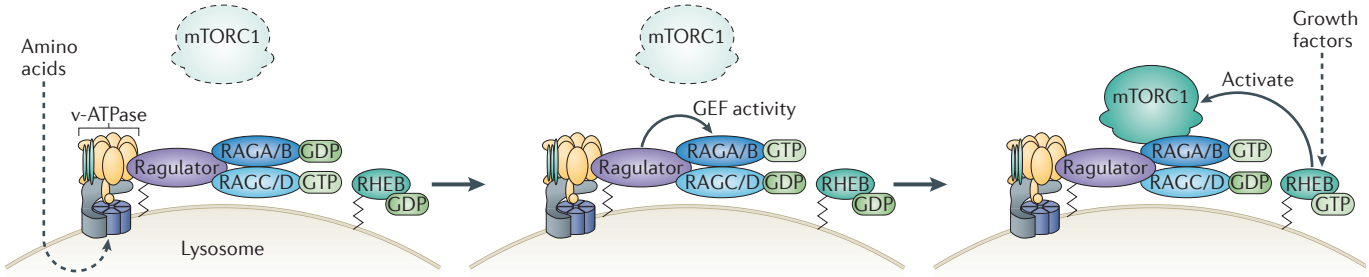


Figure 3 | **mTORC1 activation at the lysosome.** Amino acids are thought to accumulate within the lysosomal lumen and to signal to vacuolar H^+-ATPase (v-ATPase) through an 'inside–out' mechanism. v-ATPase controls RAG GTPase–Ragulator binding, and therefore Ragulator guanine exchange factor (GEF) activity and RAGA and RAGB guanine nucleotide loading (RAGA/B·GTP). The active RAG complex (RAGA/B·GTP–RAGC/D·GDP) binds to mammalian target of rapamycin complex 1 (mTORC1) and recruits it to the lysosome, through an unknown mechanism, possibly in close proximity to RHEB (RAS homologue enriched in brain). Downstream of growth factor signalling, GTP-bound RHEB potently activates mTORC1.

However, it has been observed that overexpression of the lysosome-localized amino acid transporter PAT1 (proton-assisted transporter 1), which is known to export amino acids out of the lysosome, inhibits mTORC1 activation[34]. Moreover, lysosomal membrane permeabilization, which allows amino acids to escape, blocked RAG GTPase–mTORC1 interaction[34]. Together, these observations suggest that the build-up of amino acids within the lysosomal lumen seems to be required for downstream mTORC1 activation.

Whether the amino acids that are transported into the lysosome originate from within the cell or are transported from the extracellular environment remains unclear. However, an increase in intracellular amino acid concentrations mediated by the protein synthesis inhibitor cycloheximide enhanced mTORC1 activity, which indicates that intracellular amino acids can accumulate within the lumen of the lysosome[34].

On the basis of these findings, it has been proposed that amino acids, whether intracellular or extracellular, accumulate within the lysosomal lumen and signal to v-ATPase. This enhances the GEF activity of Ragulator, promoting the active RAG GTPase conformation (RAGA/B·GTP–RAGC/D·GDP), which can then recruit mTORC1 to the lysosome, ultimately leading to mTORC1 activation. Whether v-ATPase has a role in amino acid sensing and TOR activation in yeast remains to be determined.

Activation by leucyl-tRNA synthetase

Two independent groups showed in mammals and yeast that leucyl-tRNA synthetase (LeuRS) acts as a direct sensor for the amino acid Leu and is involved in the activation of mTORC1, although the mechanistic details differ considerably between the two studies[38,39] (FIG. 2). LeuRS is a cytoplasmic enzyme that catalyses the ATP-dependent ligation of L-Leu to its corresponding tRNA and is required for protein synthesis. LeuRS has previously been reported to be a key amino acid sensor upstream of mTOR[40]. In mammals, in response to Leu, LeuRS was found to translocate to the lysosomal membrane, where it bound to and acted as a GAP for RAGD. LeuRS interacted via its C terminus with the C terminus of RAGD, which is likely to be important for heterodimerization with RAGA/B based on the structure of Gtr1–Gtr2 (REF. 38) (see above). Surprisingly, the Arg residue in human LeuRS that is required for the proposed GAP activity is not conserved in the *D. melanogaster* LeuRS homologue, which is unexpected because amino acid-induced mTORC1 activation is highly conserved in all eukaryotes.

In yeast, LeuRS was shown to regulate the activation of Gtr1 (the RAGA/B homologue), in this case by blocking GTP hydrolysis rather than promoting it. Specifically, the LeuRS editing domain (which hydrolyses mischarged tRNA Leu) interacts with GTP-bound Gtr1 and blocks GTP hydrolysis, and this interaction is facilitated by a conformational change in LeuRS triggered by binding of Leu. The domain in LeuRS that interacts with Gtr1·GTP is different from the one suggested to mediate the interaction with RAGD in mammals. It was proposed that the binding between LeuRS and Gtr1·GTP blocks the access of an unidentified GAP, thus inhibiting GTP hydrolysis[39].

Further work is needed to verify the exact mechanism of action of LeuRS in this context and also to confirm that it does indeed have a role in mTORC1 activation. Also, in contrast to the amino acid sensing inside–out model, LeuRS detects the availability of Leu in the cytoplasm. It will be important to further examine how the two mechanisms (the lysosomal inside–out amino acid sensing mechanism involving v-ATPase and the cytoplasmic amino acid sensing mechanism involving LeuRS) are integrated. Radioactively labelled amino acids accumulate in the lysosome within 10 minutes of addition, whereas mTORC1 has been reported to be activated after only 3 minutes[5,34]. It is still not clear whether amino acids are required to accumulate within the lysosome before mTORC1 is being recruited there, or if there is also a parallel signal, such as the one mediate by LeuRS, aiding mTORC1 activation.

Conclusions and perspectives

Recent work identifying new lysosomal components has provided a model whereby amino acids that have accumulated in the lysosome signal to mTORC1 through an inside–out mechanism. Moreover, new work involving LeuRS suggests that mTORC1 may also be activated by amino acids in the cytoplasm[38,39]. Although the precise amino acid sensor at the lysosome is still unknown, the first downstream target discovered thus far is v-ATPase[34]. v-ATPase promotes the GEF activity of Ragulator, resulting in RAG GTPase nucleotide exchange and activation[24]. The activated RAGA/B·GTP–RAGC/D·GDP complex binds to and recruits mTORC1 from an undefined cellular location to the lysosome, possibly in close proximity to the potent mTORC1 activator RHEB[5,20]. Amino acids are thought to relay messages to RAG GTPases, which directly bind to and redistribute mTORC1 to lysosomes. RHEB, which is activated by growth factors downstream of TSC, is also thought to reside on the lysosome. Together, these stimuli coordinate to activate mTORC1 and thus promote cell growth and inhibit autophagy. This model demonstrates how multiple stimuli may coordinately activate mTORC1 at the lysosome.

The identification of Ragulator as the RAGA/B GEF is an important advance, although the GAP has yet to be found. Future work is now needed to identify other components of the pathway. The further characterization of mTORC1 negative regulators, such as SH3 domain-binding protein 4 (SH3BP4), PAT1 and the small GTPases ARF and RAB5, will provide important insight[23,34,41]. The precise amino acid sensor and its location have yet to be identified. If the sensor is located in the lysosomal lumen, is the acidification of the lysosome required for its activation? Indeed, the functions of many lumenal proteins depend on an acidic pH. Furthermore, hydrolysis of ATP by the V_1 subunit of v-ATPase promotes acidification of the lysosome and activation of mTORC1 through undefined mechanisms. Other reports have shown that an increase in cytoplasm acidity inhibits mTORC1 (REFS 36,37). Although this is consistent with v-ATPase having a role in mTORC1 activation, is it lysosomal acidification or the pH of the cytoplasm that regulates mTORC1? Future work will undoubtedly be focused on understanding these mechanisms and how v-ATPase is regulated. For example, signals that regulate v-ATPase could potentially modulate mTORC1 activity and cell growth. It will also be important to understand how this pathway integrates with the one that is activated by LeuRS in the cytoplasm.

If the inside–out mechanism holds true and amino acids accumulate within the lysosome to signal to mTORC1, where are the amino acids coming from? Are intracellular or extracellular amino acid pools filling the lumen of the lysosome? How are the amino acids being transported into the lysosome? The identification of the lysosomal amino acid transporter will be a significant advancement.

Moreover, the precise details or unidentified intermediates involved in mTORC1 translocation to the lysosome require further investigation. Do RAG GTPases dissociate from the lysosome, perhaps in response to amino acids, and shuttle mTORC1 to the lysosome? Or are there additional, unidentified intermediates that translocate mTORC1 to the RAG GTPases at the lysosome in response to amino acids?

Amino acids are fundamental to life and essential in facilitating mTORC1 activation. Therefore, determining the molecular mechanisms involved in mTORC1 activation will lead to a better understanding of cell growth and autophagy, enhance our basic understanding of biology and also clarify the role of this pathway in disease. For example, although numerous oncogenes and tumour suppressors have been identified in the mTORC1-activating pathway involving TSC, such proteins have yet to be characterized in the amino acid cascade. Identification of additional regulators could shed light onto the role of this signalling pathway in human pathogenesis, in particular cancer.

Jenna L. Jewell, Ryan C. Russell and Kun-Liang Guan are at the Department of Pharmacology and Moores Cancer Center, University of California, San Diego, La Jolla, California 92093, USA.

Correspondence to K.-L.G.
e-mail: kuguan@ucsd.edu

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Competing interests statement
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